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(54) Title: NEUROPROTECTIVE METHODS AND REAGENTS (57) Abstract One aspect of the present application relates to a method for limiting damage to neuronal cells by ischemic or epoxic conditions, e.g., such as may be manifest by a reduction in brain infarct volume, by administering to an individual a hedgehog therapeutic or ptc therapeutic in an amount effective for reducing cerebral infarct volume.		

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Neuroprotective Methods and Reagents

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Background of the Invention

Stroke kills more than 150,000 people annually and accounts for about one of every 15 U.S. deaths. It is presently the third largest cause of death, ranking behind diseases of the heart and cancer, according to the National Center for Health Statistics.

10 On average, someone suffers a stroke in the United States every minute; every 3.4 minutes someone dies of a stroke. Based on the Framingham Heart Study, approximately 500,000 people suffer a new or recurrent brain attack each year. Approximately 3,890,000 stroke survivors are alive today. From 1984 to 1994, the death rate from stroke declined 19.8 percent, but the actual number of deaths from brain attack rose slightly.

15 Stroke is the leading cause of serious, long-term disability in the United States. Stroke accounts for half of all patients hospitalized for acute neurological disease. In 1991-92 one million Americans age 15 and older had disabilities resulting from stroke. According to the Framingham Heart Study, 31 percent of brain attack survivors needed help caring for themselves; 20 percent needed help walking; and 71 percent had an impaired ability to work when examined an average of seven years later. Sixteen percent had to be institutionalized.

20 About 31 percent of people who have an initial stroke die within a year. This percentage is higher among people older than age 65. About two-thirds of men and women who have a brain attack die within 12 years; long-term survivorship is worse in men than in women. 407,000 males and 478,000 females were discharged from hospitals in 1994 after having a stroke. For statistics, see for example the homepage for the American Heart Association at

25 <http://www.amhrt.org/1997/stats/Stroke.html>

Stroke is defined as a sudden impairment of body functions caused by a disruption in, e.g., the supply of blood to the brain. For instance, a stroke occurs when a blood vessel bringing oxygen and nutrients to the brain is interrupted by any method including low blood pressure, clogging by atherosclerotic plaque, a blood clot, or some other particle, or bursts.

30 Because of the blockage or rupture, part of the brain fails to get the blood flow that it requires. Brain tissue that receives an inadequate supply of blood is said to be ischemic. Deprived

of oxygen and nutrients, nerve cells and other cell types within the brain begin to fail, creating an infarct (an area of cell death, or necrosis). As nerve cells (neurons) fail and die, the part of the body controlled by those neurons cannot function either. The devastating effects of ischemia are often permanent because brain tissue has very limited repair capabilities and lost neurons are not usually replaced.

Cerebral ischemia may be incomplete (blood flow is reduced but not entirely cut off), complete (total loss of tissue perfusion), transient or permanent. If ischemia is incomplete and persists for no more than ten to fifteen minutes, neural death might not occur. More prolonged or complete ischemia results in infarction. Depending on the site and extent of the infarction, mild to severe neurological disability or death will follow. Thus, the chain of causality leading to neurological deficit in stroke has two principal components: loss of blood supply, and cell damage and death.

Thrombosis is the blockage of an artery by a large deposit that usually results from the combination of atherosclerosis and blood clotting. Thrombotic stroke (also called cerebral thrombosis) results when a deposit in a brain or neck artery reaches occlusive proportions. Most strokes are of this type.

Embolism is the blockage of an artery or vein by an embolus. Emboli are often small pieces of blood clot that break off from larger clots. Embolic stroke (also called cerebral embolism) occurs when an embolus is carried in the bloodstream to a brain or neck artery. If the embolus reaches an artery that is too small for it to pass through, it plugs the artery and cuts off the blood supply to downstream tissues. Embolic stroke is the clinical expression of this event.

To a modest extent, the brain is protected against cerebral ischemia by compensatory mechanisms that include: collateral circulation (overlapping local blood supplies), and arteriolar auto-regulation (local smooth muscle control of blood flow in the smallest arterial channels). If compensatory mechanisms operate efficiently, slightly diminished cerebral blood flow produces neither tissue ischemia nor abnormal signs and symptoms. Usually, such mechanisms must act within minutes to restore blood flow if permanent infarction damage is to be avoided or reduced. Arteriolar auto-regulation works by shunting blood from noncritical regions to infarct zones.

Even in the face of systemic hypotension, auto-regulation may be sufficient to adjust the circulation and thereby preserve the vitality and function of brain tissue. Alternatively, ischemia may be sufficiently prolonged and compensatory mechanisms sufficiently inadequate that a

catastrophic stroke results. With these as the extremes, the gradation of ischemic stroke are described below.

5 A transient ischemic attack (TIA) is conventionally described as a loss of neurologic function caused by ischemia, abrupt in onset, persisting for less than 24 hours, and clearing without residual signs. Most TIAs last only a few minutes. However, neurologic disability may persist for more than 24 hours before clearing, such an event is called a reversible ischemic neurological disability (RIND).

10 An ischemic event that is sufficiently severe to cause persistent disability but that is short of a calamitous stroke, is called a partial nonprogressing stroke (PNS). The penultimate ischemic event, a completed stroke, produces major functional loss. The ultimate ischemic insult is death.

Focal cerebral ischemia must be distinguished from global cerebral hypoxia. In cerebral hypoxia the oxygen supply to the brain is diminished even though blood flow and blood pressure may be normal. Discriminating between diagnoses of patients with acute neurological deficit is critical because patient management takes disparate paths.

15 There are generally distinct clinical outcomes in stroke versus cerebral hypoxia, although both sets of patients may suffer death or permanent damage. Hypoxia patients who survive past an acute life-threatening period usually show few immediate symptoms of long term damage. Instead, clinical manifestations such as mental deterioration, urinary and fecal incontinence, gait and speech disturbances, tremor and weakness are delayed for periods that may vary from days to weeks. However, as in stroke, progressive loss of neurons due to oxygen deprivation is
20 believed to be a factor in such detrimental effects of hypoxia.

It is an objective of the present application to provide new drugs for treatment and prophylaxis of cerebral ischemia, such as stroke.

25 It is also an objective of the present application to provide new drugs for treatment and prophylaxis of cerebral hypoxia.

Summary of the Invention

One aspect of the present application relates to a method for limiting damage to neuronal cells by ischemic or epoxic conditions, e.g., such as may be manifest by a reduction in brain
30 infarct volume, by administering to an individual a hedgehog therapeutic or ptc therapeutic in an

amount effective for reducing cerebral infarct volume relative to the absence of administration of the hedgehog therapeutic or *ptc* therapeutic.

5 In other embodiments, the subject method can be used for protecting cerebral tissue of a mammal against the repercussions of ischemia; for treating cerebral infarctions; for treating cerebral ischemia; for treatment of stroke; and/or for treating transient ischemia attacks. In embodiments wherein the patient is treated with a *ptc* therapeutic, such therapeutics are preferably small organic molecules which mimic *hedgehog* effects on *patched*-mediated signals.

Wherein the subject method is carried out using a *hedgehog* therapeutic, the *hedgehog* therapeutic preferably a polypeptide including a *hedgehog* portion comprising at least a bioactive
10 extracellular portion of a *hedgehog* protein, e.g., the *hedgehog* portion includes at least 50, 100 or 150 amino acid residues of an N-terminal half of a *hedgehog* protein. In preferred embodiments, the *hedgehog* portion includes at least a portion of the *hedgehog* protein corresponding to a 19kd fragment of the extracellular domain of a *hedgehog* protein.

In preferred embodiments, the *hedgehog* portion has an amino acid sequence at least 60,
15 75, 85, or 95 percent identical with a *hedgehog* protein of any of SEQ ID Nos. 10-18, though sequences identical to those sequence listing entries are also contemplated as useful in the present method. The *hedgehog* portion can be encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid sequence of any of SEQ ID Nos. 1-9, e.g., the *hedgehog* portion can be encoded by a vertebrate *hedgehog* gene, especially a human *hedgehog* gene.

20 In other embodiments, the subject method can be carried out by administering a gene activation construct, wherein the gene activation construct is designed to recombine with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.

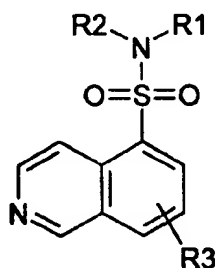
In still other embodiments, the subject method can be practiced with the administration of a gene
25 therapy construct encoding a *hedgehog* polypeptide. For instance, the gene therapy construct can be provided in a composition selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent,

Where the subject method is carried out using a *ptc* therapeutic, the therapeutic can be, e.g., a molecule which binds to *patched* and mimics *hedgehog*-mediated *patched* signal
30 transduction. For instance, the binding of the therapeutic to *patched* may result in upregulation of *patched* and/or *gli* expression.

In other embodiments, the *ptc* therapeutic mimics *hedgehog*-mediated *patched* signal transduction by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway.

In preferred embodiment, the *ptc* therapeutic is a small organic molecule, e.g., less than 5 kD, more preferably less than 2.5 kD. For instance, the present invention contemplates the use of small organic molecules which interact with neuronal cells to mimic *hedgehog*-mediated *patched* signal transduction.

In a preferred embodiment, the *ptc* therapeutic is a PKA inhibitor. A variety of PKA inhibitors are known in the art, including both peptidyl and organic compounds. For instance, the *ptc* therapeutic can be a 5-isoquinolinesulfonamide, such as represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

R₈ represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

Exemplary PKA inhibitor of this class include N-[2-((p-bromocinnamyl)amino)ethyl]-5-
5 isoquinoline-sulfonamide and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine. Other PKA
inhibitors which can be used in the subject method include KT5720; cyclic AMP analogs (such
as 8-bromo-cAMP or dibutyryl-cAMP); and PKA Heat Stable Inhibitor (isoform α).

In yet other embodiments of the present invention, the *ptc* therapeutic alters the level of
expression of a *hedgehog* protein, a *patched* protein or another protein involved in the
10 intracellular signal transduction pathway of *patched*. In this regard, the *ptc* therapeutic can be an
antisense construct which inhibits the expression of a protein which is involved in the signal
transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated
signals. For example, the antisense molecule can be one which hybridizes to a *patched* transcript
or genomic sequence, such as 5'-GTCCTGGCGCCGCCGCCGCCGTCGCC, 5'-TTCCGATG-
15 ACCGGCCTTTTCGCGGTGA or 5'-GTGCACGGAAAGGTGCAGGCCACACT.

In yet other embodiments, the subject method can be carried out with a gene activation
construct, which construct recombines with a genomic *hedgehog* gene of the patient, e.g., to
form a chimeric gene, providing a heterologous transcriptional regulatory sequence operatively
linked to a coding sequence of the *hedgehog* gene. The transcriptional regulatory sequence can
20 provide for constitutive or inducible expression of the *hedgehog* gene.

The subject method can be used as part of a treatment for stroke, e.g., thrombotic stroke
and/or embolic stroke

The subject method can also be used to treat hypoxic conditions which otherwise result
in cerebral hypoxia.

25 The subject method can be used prophylactically or as an ipso facto treatment. It can be
used to treat patients who are hypotensive.

The subject method can also be used as part of a therapy including administering one or
more of an anticoagulation, an antiplatelet agent, a thrombin inhibitors, and/or a thrombolytic
agent, and/or in conjunction with vascular surgery, e.g., carotid endarterectomy.

In preferred embodiments, the subject method results in at least a 25%, 50%, 70%, 75%, or 90% reduction in cerebral infarct volumes relative to absence of treatment with the therapeutic, e.g., as measured in a stroke model such as the MCAO model.

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Brief Description of the Drawings

Figure 1 is a graph demonstrating the effect of systemic *hedgehog* treatment on cerebral infarction volume in rat models of middle cerebral artery occlusion.

10

Detailed Description of the Invention

Stroke occurs when the flow of oxygen and nutrients to the brain is inhibited/interrupted due to any cause. Thus, in certain indications, stroke is a form interrupted of cardiovascular disease that affects the arteries of the central nervous system. For example, a stroke occurs when a blood vessel bringing oxygen and nutrients to the brain bursts or is clogged by a blood clot or some other particle. Because of this rupture or blockage, part of the brain doesn't get the flow of blood it needs. Deprived of oxygen, nerve cells in the affected area of the brain can't function and die within minutes. Depending on the part of the brain affected by the brain attack/stroke, there may be loss of normal function. Strokes are the third most common cause of death in United States. Stroke is the most common cause of disability of all conditions in adults.

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In terms of treatment, once a patient experiences symptoms of a transient ischemic attack, the goal of therapy is prevention of stroke. If a stroke occurs, the goal of therapy changes to the limiting of damage. Preventing stroke and limiting the damage of stroke are currently carried out in the art through medication or surgery. In both cases, the treatment involves reducing or removing blocks, building up in blood vessels and preventing further cell death about neuronal populations. These treatments include the use of (a) anticoagulations, (b) antiplatelet agents, and (c) vascular surgery. For instance, anticoagulation drug therapy inhibits the coagulation process. Heparin, which inhibits enzymes and platelets that causes clots, is used in acute settings. For long term prevention, warfarin offers anticoagulation by stopping production of Vitamin K dependent coagulation factors. With both drugs, there runs a risk of hemorrhage and is only used for ischemic strokes. Strokes involving certain areas also do not warrant this therapy. Another therapy known in the art, antiplatelet therapy with aspirin, provides one of the

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most important preventive tools available. At low daily doses, aspirin has been shown to reduce the incidence of stroke. Specifically, low doses of aspirin block the production of a chemical called thromboxane. Thromboxane's function is to activate platelets to bind together and thus form blood clots. Finally, carotid endarterectomy is the surgical procedure where the plaque at the origin of the carotid artery is removed. This is the treatment of choice of patients with TIA's caused by embolism, low flow, and with minor strokes due to narrowing greater than 70% of the internal carotid.

I. Overview

The present application is directed to compositions and methods for the prevention and treatment of ischemic injury to the brain, such as resulting from stroke. The invention derives, at least in part, from the observation of a protective effect by the so called "*hedgehog*" proteins on animal stroke models. Briefly, as described in the appended examples, we investigated the neuroprotective potential of *hedgehog* proteins in a rat model of focal cerebral ischemia that used permanent occlusion of the middle cerebral artery. Intravenous infusion of vehicle (control) or *Shh* (sonic *hedgehog*) was administered for 3 hours beginning 30 minutes after occlusion, and resulted in about a 70 percent reduction in total infarct size ($P=0.0039$), relative to the control, when examined 24 hours post-occlusion. Measurements of arterial blood pressure, blood gases, glucose, hematocrit and osmolality revealed no difference among vehicle- and *Shh*-treated animals. These results show that the intravenous *hedgehog* protein reduces neuronal damage due to stroke. There was no apparent cytotoxicity associated with administration of the *hedgehog* polypeptide.

These results, in comparison to neuroprotective agents described in the art, suggest an unexpectedly good neuroprotective activity for *hedgehog* in the treatment of stroke. For example, the non-competitive antagonist of the NMDA receptor, MK-801, was typically reported to produce less than a 50% reduction in infarct volume. Work on MK-801 was halted because of significant safety concerns, mostly related to vacuolization seen in neurons of animal models. Moreover, MK-801 has a relatively short therapeutic window and must be given within a few hours of the ischemic attack.

Another neuroprotective agent presently being investigated for use in the treatment of stroke is basic fibroblast growth factor (bFGF). In one study, (Tatlisumak et al. (1996) *Stroke* 27:2292), bFGF (45 $\mu\text{g/kg/hr}$) or vehicle was infused intravenously for three hours beginning 30

minutes after permanent middle cerebral artery occlusion by intraluminal suture in mature Sprague-Dawley rats. After 24 hours, neurological deficit and infarct volume were significantly improved (approximately 50% reduction in infarct volume) in the FGF group. Autoradiography following intravenous administration of radiolabeled bFGF showed that labeled FGF (confirmed by immunoprecipitation) crossed the damaged blood brain barrier to enter the ischemic, but not the non-ischemic hemisphere.

A second model (Jiang et al. (1995) *Stroke* 26:1-40), utilized mature Wistar rats which underwent temporary occlusion of the middle cerebral artery by intra-arterial suture for two hours. At the time of reperfusion either bFGF (45 µg/kg/hr) or vehicle were infused intravenously over three hours. At seven days after ischemia, infarct volume was significantly reduced in the bFGF treated animals (approximately 40% reduction in infarct volume), and only the bFGF treated animals regained their weight after surgery.

In one aspect, the present invention provides pharmaceutical preparations and methods for preventing/treating cerebral ischemia and the like utilizing, as an active ingredient, a *hedgehog* polypeptide or a mimetic thereof.

The subject *hedgehog* treatments are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs and goats.

However, without wishing to be bound by any particular theory, the reduction in infarct size in the present studies may be due at least in part to the ability of *hedgehog* proteins to antagonize (directly or indirectly) *patched*-mediated regulation of gene expression and other physiological effects mediated by the *patched* gene. The *patched* gene product, a cell surface protein, is understood to signal through a pathway which regulates transcription of a variety of genes involved in neuronal cell development. In the CNS and other tissue, the introduction of *hedgehog* relieves (derepresses) this inhibition conferred by *patched*, allowing expression of particular gene programs.

Accordingly, the present invention contemplates the use of other agents which are capable of mimicking the effect of the *hedgehog* protein on *patched* signalling, e.g., as may be identified from the drug screening assays described below.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

5 A "stroke" is a sudden loss of function caused by a cutoff in the blood supply to the brain. Stroke presents with different levels of severity ranging from "transient ischemic attack" or "TIA" (no permanent disability), to "partial nonprogressing stroke" (persistent but no calamitous damage), to "complete stroke" (permanent, calamitous neurological deficit). Ischemia (diminished or stopped blood flow) and infarction (cell damage and death within the zone of
10 ischemia) are the pathologic processes in stroke that lead to neurologic deficits.

"Ischemic stroke" is caused by an obstruction of blood vessels supplying the brain. The primary subcategories of ischemic stroke are thrombotic stroke, embolic stroke and lacunar infarctions.

"Hemorrhagic stroke" is caused by the rupture of blood vessels supplying the brain. The
15 primary subcategories of hemorrhagic stroke are subarachnoid hemorrhage (SAH) and intracerebral hemorrhage (ICH).

The term "ischemic damage" refers to a reduction in the biological capability of a neuronal cell, including cell death, induced by a reduced blood flow, or an otherwise reduced level of oxygen to the affected neuronal cells, whether it be the result of ischemic stroke,
20 hemorrhagic stroke, hypoxia or the like.

The term "*hedgehog* therapeutic" refers to various forms of *hedgehog* polypeptides, as well as peptidomimetics, which are neuroprotective for neuronal cells, and in particular, enhance the survival of neurons under ischemic and/or epoxic conditions. These include naturally occurring forms of *hedgehog* proteins, as well as modified or mutant forms generated by
25 molecular biological techniques, chemical synthesis, etc. While in preferred embodiments the *hedgehog* polypeptide is derived from a vertebrate homolog, cross-species activity reported in the literature supports the use of *hedgehog* polypeptides from invertebrate organisms as well. Naturally and non-naturally occurring *hedgehog* therapeutics referred to herein as "agonists" mimic or potentiate (collectively "agonize") the effects of a naturally-occurring *hedgehog* protein
30 as a neuroprotective agent. In addition, the term "hedgehog therapeutic" includes molecules which can activate expression of an endogenous *hedgehog* gene. The term also includes gene

therapy constructs for causing expression of *hedgehog* polypeptides *in vivo*, as for example, expression constructs encoding recombinant *hedgehog* polypeptides as well as trans-activation constructs for altering the regulatory sequences of an endogenous *hedgehog* gene by homologous recombination.

5 In particular, the term "*hedgehog* polypeptide" encompasses *hedgehog* proteins and peptidyl fragments thereof.

As used herein the term "bioactive fragment", with reference to a portions of *hedgehog* proteins, refers to a fragment of a full-length *hedgehog* protein, wherein the fragment specifically agonizes neuroprotective events mediated by wild-type *hedgehog* proteins. The *hedgehog*
10 bioactive fragment preferably is a soluble extracellular portion of a *hedgehog* protein, where solubility is with reference to physiologically compatible solutions. Exemplary bioactive fragments are described in PCT publications WO 95/18856 and WO 96/17924.

The term "ptc therapeutic" refers to agents which mimic the effect of naturally occurring *hedgehog* proteins on *patched* signalling. The ptc therapeutic can be, e.g., a peptide, a nucleic
15 acid, a carbohydrate, a small organic molecule, or natural product extract (or fraction thereof).

A "patient" or "subject" to be treated by the subject method are mammals, including humans.

A "therapeutically effective amount" of, e.g., a *hedgehog* or ptc therapeutic, with respect to the subject method of treatment, refers to an amount of the therapeutic (in a preparation)
20 which when applied as part of a desired dosage regimen causes a decrease in ischemia- and/or hypoxia-induced neuronal cell death (i.e. a reduction in the volume/size of a cerebral infarct caused thereby) according to clinically acceptable standards for the treatment or prevention of those disorder.

By "protection from damage to neural tissue" it is meant reduction in the total stroke
25 volume and/or infarct volume resulting from, e.g., ischemic or hypoxic conditions, preferably as manifested by less neurological and/or cognitive deficits.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for
30 purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when

the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An
5 "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an AR sequence of the present invention.

The term "corresponds to", when referring to a particular polypeptide or nucleic acid sequence is meant to indicate that the sequence of interest is identical or homologous to the reference sequence to which it is said to correspond.

10 The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression construct which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

15 A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a *hedgehog* polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of *hh* protein. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein
20 structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula $(X)_n-(hh)_m-(Y)_n$, wherein *hh* represents all or a portion of the *hedgehog* protein. X and Y each independently represent an amino acid sequences which are not naturally found as a polypeptide chain contiguous with the *hedgehog* sequence. m is an integer greater than or equal to 1, and each occurrence of n is, independently, 0 or an integer greater than
25 or equal to 1 (n and m are preferably no greater than 5 or 10).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. The term "expression vector" includes plasmids, cosmids or phages capable of synthesizing, for example, the subject *hedgehog* polypeptides encoded by the respective recombinant gene carried by the vector. Preferred
30 vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. Moreover, the invention is intended to

include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, as well as polyadenylation sites, which induce or control transcription of protein (or antisense) coding sequences with which they are operably linked. In preferred embodiments, transcription of a recombinant gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of the regulatory protein.

The term "operably linked" refers to the arrangement of a transcriptional regulatory element relative to other transcribable nucleic acid sequence such that the transcriptional regulatory element can regulate the rate of transcription from the transcribable sequence(s).

III. Exemplary Applications of Method and Compositions

Central nervous system tissue is particularly vulnerable to damage caused by ischemic conditions. The subject method has wide applicability to the treatment or prophylaxis of ischemic or hypoxic damage marked by neuronal cell death. The instant treatment can be used to treat or prevent injury or disease to brain tissue resulting from ischemia, e.g., as caused from insufficient oxygen. The types of ischemia for which the subject method can be used as part of a treatment include, but are not limited to those which may last for only transient periods of time to those which may last for lengthy durations, as in stroke. In the regard, the subject method is useful for treatment and prevention of injury to the brain and spinal cord and edema due to head trauma, spinal trauma, stroke, hypotension, arrested breathing, cardiac arrest, Rey's syndrome, cerebral thrombosis, embolism, hemorrhage or tumors, encephalomyelitis, hydroencephalitis, and operative and postoperative brain injury.

In general, the method can be characterized as including a step of administering to an animal an amount of a *ptc* or *hedgehog* therapeutic effective to enhance the survival of neuronal cells under such ischemic or hypoxic conditions. The mode of administration and dosage

regimens will vary depending on the severity of the ischemic or hypoxic attack, e.g., the dosage may be altered as between a transient ischemic attack, a partial nonprogressing stroke, and a complete stroke. In preferred embodiments, the *ptc* or *hedeghog* therapeutic is administered systemically initially (i.e., while the blood brain barrier is disrupted), then locally for medium to
5 long term care.

When used to treat stroke, the clinician should not only define the level of stroke severity, but also the "pace" or "tempo" of the illness. This is because the pace of progression helps to dictate the urgency for evaluation and treatment. A patient who suffers a TIA in the morning has a higher risk for stroke in the afternoon than a patient who suffered a single TIA a
10 month earlier. Where the risk of stroke remains high, the subject *hedeghog* and *ptc* therapeutics can be used prophylactically in order to minimize ischemic damage which may result from an eventual stroke. A patient who is worsening under supervision requires more urgent management than one who has been stable for a week or more.

The subject method may also find particular utility in treating or preventing the adverse
15 neurological consequences of surgery. For example, coronary bypass surgery requires the use of heart-lung machines, which tend to introduce air bubbles into the circulatory system that may lodge in the brain. The presence of such air bubbles robs neuronal tissue of oxygen, resulting in anoxia and ischemia. Pre- or post-surgical administration of the *hedeghog* and/or *ptc* therapeutics of the present invention will treat or prevent the resulting ischemia. In a preferred embodiment,
20 the subject therapeutics are administered to patients undergoing cardiopulmonary bypass surgery or carotid endarterectomy surgery.

In still other embodiments, the subject method can be used in the prevention and/or treatment of hypoxia, e.g., as a neuroprotective agent. For instance, the subject method can be used prophylactically to lessen the neuronal cell death caused by altitude-induced hypoxia.

25 A method which is "neuroprotective", in the case of cerebral ischemia, results in diminished infarct volume relative to that which would occur in the absence of treatment with a *hedeghog* or *ptc* therapeutic. That is a neuroprotective therapy is intended to maintain or rescue damaged nerve cells, preventing their death.

The treatment methods of the present invention can be combined with the use of (a)
30 anticoagulations, (b) antiplatelet agents, and/or (c) vascular surgery. Co-administered with suitable anti-coagulant agents, antiplatelet agents, thrombin inhibitors, and/or thrombolytic agents, may afford an efficacy advantage over any of the agents alone, and may do so while

permitting the use of lower doses of each. A lower dosage minimizes the potential of side effects, thereby providing an increased margin of safety. The two (or more) agents are administered in combination according to the invention. The term "in combination" in this context means that the drugs are given substantially contemporaneously, either simultaneously or sequentially. If given sequentially, at the onset of administration of the second agent, the first of the two agents is preferably still detectable at effective concentrations at the site of treatment.

The term anti-coagulant agents (or coagulation inhibitory agents), as used herein, denotes agents that inhibit blood coagulation. Such agents include warfarin, heparin, or low molecular weight heparin (LMWH), including pharmaceutically acceptable salts or prodrugs thereof. For reasons of efficacy, the preferable anti-coagulant agents are warfarin or heparin or LMWH. The warfarin employed herein, may be, for example, crystalline warfarin or amorphous sodium warfarin. The heparin employed herein may be, for example, the sodium or sulfate salts thereof.

The term anti-platelet agents (or platelet inhibitory agents), as used herein, denotes agents that inhibit platelet function such as by inhibiting the aggregation, adhesion or granular secretion of platelets. Such agents include the various known non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen, naproxen, sulindac, indomethacin, mefenamate, droxicam, diclofenac, sulfinpyrazone, and piroxicam, including pharmaceutically acceptable salts or prodrugs thereof. Of the NSAIDS, aspirin (acetylsalicylic acid or ASA), which has been well researched and widely used with good results, and piroxicam, which exerts its anti-platelet effect when dosed once daily, are preferred compounds, especially aspirin. Piroxicam is commercially available from Pfizer Inc. (New York, NY), as FELDANE TM. Other suitable anti-platelet agents include ticlopidine, including pharmaceutically acceptable salts or prodrugs thereof. Ticlopidine is also a preferred compound since it is known to be gentle on the gastrointestinal tract in use. Still other suitable platelet inhibitory agents include thromboxane-A₂-receptor antagonists and thromboxane-A₂-synthetase inhibitors, as well as pharmaceutically acceptable salts or prodrugs thereof.

The phrase thrombin inhibitors (or anti-thrombin agents), as used herein, denotes inhibitors of the serine protease thrombin. By inhibiting thrombin, various thrombin-mediated processes, such as thrombin-mediated platelet activation (that is, for example, the aggregation of platelets, and/or the granular secretion of plasminogen activator inhibitor-1 and/or serotonin) and/or fibrin formation are disrupted. Such inhibitors include boro-peptides, hirudin and argatroban, including pharmaceutically acceptable salts and prodrugs thereof. Preferably the thrombin inhibitors are boro-peptides. By boro-peptides, it is meant, N-acetyl and peptide

derivatives of boronic acid, such as C-terminal alpha -aminoboronic acid derivatives of lysine, ornithine, arginine, homoarginine and corresponding isothiuronium analogs thereof. The term hirudin, as used herein, includes suitable derivatives or analogs of hirudin, referred to herein as hirulogs, such as disulfatohirudin.

5 The phrase thrombolytics (or fibrinolytic) agents (or thrombolytics or fibrinolytics), as used herein, denotes agents that lyse blood clots (thrombi). Such agents include tissue plasminogen activator, anistreplase, urokinase or streptokinase, including pharmaceutically acceptable salts or prodrugs thereof. Tissue plasminogen activator (tPA) is commercially available from Genentech Inc., South San Francisco, Calif. The term anistreplase, as used herein,
10 refers to anisoylated plasminogen streptokinase activator complex, as described, for example, in European Patent Application No. 0 28 489, the disclosures of which are hereby incorporated herein by reference herein, in their entirety. Anistreplase is commercially available from the Beecham Group, Middlesex, England, under the trademark EMINASE TM . The term urokinase, as used herein, is intended to denote both dual and single chain urokinase, the latter also being
15 referred to herein as prourokinase.

 In yet other embodiments, the subject method can be carried out conjointly with the administration of growth and/or trophic factors. For instance, the trophic growth factor basic FGF has been demonstrated in the art to be useful in the functional recovery following experimental stroke. In experiments providing exogenous administration of bFGF after infarction, the early
20 administration of bFGF was found to reduce infarct size. See, for example, Kawamata et al. (1997) *Adv Neurol* 73: 377-82. Likewise, progesterone has been shown to be neuroprotective after transient middle cerebral artery occlusion in male rats. Jiang et al. (1996) *Brain Res* 735:101-7. Other agents with which the subject *hedgehog* and *ptc* therapeutics can be coadministered include nitro-L-arginine, transforming growth factor- β 1 (TGF-beta 1) has been
25 shown to rescue cultured neurons from excitotoxic and hypoxic cell death and to reduce infarct size after focal cerebral ischemia in mice and rabbits. In other instances, the combinatorial therapy can include a trophic factor such as nerve growth factor, ciliary neurotrophic growth factor, schwannoma-derived growth factor, glial growth factor, stiatal-derived neuronotrophic factor, platelet-derived growth factor, and scatter factor (HGF-SF). Antimitogenic agents can
30 also be used, as for example, cytosine, arabinoside, 5-fluorouracil, hydroxyurea, and methotrexate.

 Determination of a therapeutically effective amount and a prophylactically effective amount of a *hedgehog* or *ptc* therapeutic, e.g., to be adequately neuroprotective, can be readily

made by the physician or veterinarian (the "attending clinician"), as one skilled in the art, by the use of known techniques and by observing results obtained under analogous circumstances. The dosages may be varied depending upon the requirements of the patient in the judgment of the attending clinician, the severity of the condition being treated, the risk of further ischemic or hypoxic damage to the CNS, and the particular agent being employed. In determining the therapeutically effective neuroprotective amount or dose, and the prophylactically effective amount or dose, a number of factors are considered by the attending clinician, including, but not limited to: the specific cause of the ischemic or hypoxic state and its likelihood of recurring or worsening; pharmacodynamic characteristics of the particular agent and its mode and route of administration; the disorder time course of treatment; the species of mammal; its size, age, and general health; the response of the individual patient; the particular compound administered; the bioavailability characteristics of the preparation administered; the dose regimen selected; the kind of concurrent treatment (i.e., the interaction of the *hedgehog* or *ptc* therapeutic with other co-administered therapeutics); and other relevant circumstances.

Treatment can be initiated with smaller dosages which are less than the optimum dose of the agent. Thereafter, the dosage should be increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day if desired. A therapeutically effective antineoplastic amount and a prophylactically effective neuroprotective amount of a *hedgehog* polypeptide, for instance, is expected to vary from concentrations about 0.1 nanogram per kilogram of body weight per day (kg/day) to about 100 kg/day.

Potential *hedgehog* and *ptc* therapeutics, such as described below, can be tested by measuring the volume of cerebral infarction in animals receiving systemic injections. For instance, selected agents can be evaluated in the focal stroke model involving permanent middle cerebral artery occlusion (MCAO) in the spontaneously hypertensive rat. This procedure results in a reliably large neocortical infarct volume that is measured by means of vital dye exclusion in serial slices through the brain 24 hours after MCAO. Tamura et al. (1981) *J Cerebral Blood Flow and Metabolism* 1:53-60.

The middle cerebral artery is the cerebral blood vessel most susceptible to stroke in humans. In animals, coagulation, permanent ligation or permanent placement of an occluding thread in the artery produces a permanent focal stroke affecting the MCA territory. Transient ligation or occlusion results in transient focal stroke. Both transient and permanent focal strokes result in varying degrees of edema and infarction in the affected brain regions. The ability of

compounds to reduce the volumes of edema and infarction is considered a measure of their potential as anti-stroke treatment.

Compounds which are determined to be effective for the prevention or treatment of cerebral infarction and the like in animals, e.g., dogs, rodents, may also be useful in treatment of tumors in humans. Those skilled in the art of treating in such disorders in humans will be guided, from the data obtained in animal studies, to the correct dosage and route of administration of the compound to humans. In general, the determination of dosage and route of administration in humans is expected to be similar to that used to determine administration in animals.

The identification of those patients who are in need of prophylactic treatment for ischemic or hypoxic states is well within the ability and knowledge of one skilled in the art. Certain of the methods for identification of patients which are at risk of cerebral infarction which can be treated by the subject method are appreciated in the medical arts, such as family history of the development of a particular disease state and the presence of risk factors associated with the development of that disease state in the subject patient. A clinician skilled in the art can readily identify such candidate patients, by the use of, for example, clinical tests, physical examination and medical/family history.

IV. Exemplary hedgehog therapeutic compounds.

The *hedgehog* therapeutic compositions of the subject method can be generated by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the *hedgehog* therapeutics are preferably derived from vertebrate *hedgehog* proteins, e.g., have sequences corresponding to naturally occurring *hedgehog* proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the *hedgehog* polypeptide can correspond to a *hedgehog* protein (or fragment thereof) which occurs in any metazoan organism.

The various naturally-occurring *hedgehog* proteins from which the subject therapeutics can be derived are characterized by a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.* (1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353), *hedgehog* precursor proteins naturally undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 374:363-

366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart', E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of *hedgehog* encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the *hedgehog* precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is suggested that the nucleophile is a small lipophilic molecule, more particularly cholesterol, which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., paralogs of the single drosophila *hedgehog* gene (SEQ ID No. 19). Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish. According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID No:1; a mouse *Dhh* polypeptide is encoded by SEQ ID No:2; a mouse *Ihh* polypeptide is encoded by SEQ ID No:3; a mouse *Shh* polypeptide is encoded by SEQ ID No:4 a zebrafish *Shh* polypeptide is encoded by SEQ ID No:5; a human *Shh* polypeptide is encoded by SEQ ID No:6; a human *Ihh* polypeptide is encoded by SEQ ID No:7; and a zebrafish *Thh* is encoded by SEQ ID No. 8.

Table 1

Guide to *hedgehog* sequences in Sequence Listing

	Nucleotide	Amino Acid
Chicken <i>Shh</i>	SEQ ID No. 1	SEQ ID No. 10
Mouse <i>Dhh</i>	SEQ ID No. 2	SEQ ID No. 11
Mouse <i>Ihh</i>	SEQ ID No. 3	SEQ ID No. 12
Mouse <i>Shh</i>	SEQ ID No. 4	SEQ ID No. 13

Zebrafish <i>Shh</i>	SEQ ID No. 5	SEQ ID No. 14
Human <i>Shh</i>	SEQ ID No. 6	SEQ ID No. 15
Human <i>lhh</i>	SEQ ID No. 7	SEQ ID No. 16
Zebrafish <i>Thh</i>	SEQ ID No. 8	SEQ ID No. 17
Drosophila <i>HH</i>	SEQ ID No. 9	SEQ ID No. 18

In addition to the sequence variation between the various *hedgehog* homologs, the *hedgehog* proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence.

As described above, further processing of the mature form occurs in some instances to yield biologically active fragments of the protein. For instance, *sonic hedgehog* undergoes additional proteolytic processing to yield two peptides of approximately 19 kDa and 27 kDa, the 19kDa fragment corresponding to an proteolytic N-terminal portion of the mature protein. In addition to proteolytic fragmentation, the vertebrate *hedgehog* proteins can also be modified post-translationally, such as by glycosylation and/or addition of cholesterol, though bacterially produced (e.g. unglycosylated/uncholesterolized) forms of the proteins still maintain certain of the bioactivities of the native protein. Bioactive fragments of *hedgehog* polypeptides of the present invention have been generated and are described in great detail in, e.g., PCT publications WO 95/18856 and WO 96/17924.

Moreover, mutagenesis can be used to create modified *hh* polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified *hedgehog* polypeptides can also include those with altered post-translational processing relative to a naturally occurring *hedgehog* protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, the *hedgehog* therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a *hedgehog* coding sequence represented in one or more of SEQ ID Nos:1-9. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current*

Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

As described in the literature, genes for other *hedgehog* proteins, e.g., from other animals, can be obtained from mRNA or genomic DNA samples using techniques well known in the art. For example, a cDNA encoding a *hedgehog* protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a *hedgehog* protein can also be cloned using established polymerase chain reaction techniques.

Preferred nucleic acids encode a *hedgehog* polypeptide comprising an amino acid sequence at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence selected from the group consisting of SEQ ID Nos:10-18. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with an amino acid sequence represented in one of SEQ ID Nos:10-18 are also within the scope of the invention.

Hedgehog polypeptides preferred by the present invention, in addition to native *hedgehog* proteins, are at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence represented by any of SEQ ID Nos:10-18. Polypeptides which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous with a sequence selected from the group consisting of SEQ ID Nos:10-18 are also within the scope of the invention. The only prerequisite is that the *hedgehog* polypeptide is capable of protecting neuronal cells against ischemic damage.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a *hedgehog* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant *hedgehog* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *hedgehog* protein, or an amino acid

sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The method of the present invention can also be carried out using variant forms of the naturally occurring *hedgehog* polypeptides, e.g., mutational variants.

5 As is known in the art, *hedgehog* polypeptides can be produced by standard biological techniques. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide *hedgehog* may be secreted and isolated from a mixture of cells and medium containing the recombinant *hedgehog* polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal
10 peptide sequence from the recombinant *hedgehog* gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant *hedgehog* polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying
15 proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant *hedgehog* polypeptide is a fusion protein containing a domain which facilitates its purification, such as an *hedgehog*/GST fusion protein. The host cell may be any prokaryotic or eukaryotic cell.

20 Recombinant *hedgehog* genes can be produced by ligating nucleic acid encoding an *hedgehog* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject *hedgehog* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a *hedgehog* polypeptide include plasmids of the types: pBR322-derived
25 plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.*
30 (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid.

In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an *hedgehog* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the *hedgehog* genes represented in SEQ ID Nos:1-9 or 19.

5 The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of
10 these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host
15 organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

 In some instances, it may be desirable to express the recombinant *hedgehog* polypeptide
20 by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

 When it is desirable to express only a portion of a *hedgehog* protein, such as a form
25 lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757)
30 and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing *hedgehog*-derived

polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the *hedgehog* polypeptides of the present invention. For example, *hedgehog* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *hedgehog* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to replace the signal sequence which naturally occurs at the N-terminus of the *hedgehog* protein (e.g. of the pro-form, in order to permit purification of the poly(His)-*hedgehog* protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

Hedgehog polypeptides may also be chemically modified to create *hedgehog* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, cholesterol, isoprenyl, myristyl, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional

groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

For instance, *hedgehog* proteins can be generated to include a moiety, other than sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the *hedgehog* polypeptide to support attachment of the chimeric molecule to a cell through binding ECM components (Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacher et al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

In preferred embodiment, the *hedgehog* polypeptide is isolated from, or is otherwise substantially free of, other cellular proteins, especially other extracellular or cell surface associated proteins which may normally be associated with the *hedgehog* polypeptide. The term "substantially free of other cellular or extracellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure or purified preparations" are defined as encompassing preparations of *hedgehog* polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. By "purified", it is meant that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in any of SEQ ID Nos:10-18, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

With respect to bioactive fragments of *hedgehog* polypeptide, preferred *hedgehog* therapeutics include at least 50 amino acid residues of a *hedgehog* polypeptide, more preferably at least 100, and even more preferably at least 150.

Another preferred *hedgehog* polypeptide which can be included in the *hedgehog* therapeutic is an N-terminal fragment of the mature protein having a molecular weight of approximately 19 kDa.

Preferred human *hedgehog* proteins include N-terminal fragments corresponding approximately to residues 24-197 of SEQ ID No. 15 and 28-202 of SEQ ID No. 16. By "corresponding approximately" it is meant that the sequence of interest is at most 20 amino acid residues different in length to the reference sequence, though more preferably at most 5, 10 or 15 amino acid different in length.

Still other preferred *hedgehog* polypeptides includes an amino acid sequence represented by the formula A-B wherein: (i) A represents all or the portion of the amino acid sequence designated by residues 1-168 of SEQ ID No:19; and B represents at least one amino acid residue of the amino acid sequence designated by residues 169-221 of SEQ ID No:19; (ii) A represents all or the portion of the amino acid sequence designated by residues 24-193 of SEQ ID No:15; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:15; (iii) A represents all or the portion of the amino acid sequence designated by residues 25-193 of SEQ ID No:13; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:13; (iv) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No:11; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:11; (v) A represents all or the portion of the amino acid sequence designated by residues 28-197 of SEQ ID No:12; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:12; or (vi) A represents all or the portion of the amino acid sequence designated by residues 29-197 of SEQ ID No:16; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:16. In certain preferred embodiments, A and B together represent a contiguous polypeptide sequence designated sequence. A represents at least 25, 50, 75, 100, 125 or 150 amino acids of the designated sequence, and B represents at least 5, 10, or 20 amino acid residues of the amino acid sequence designated by corresponding entry in the sequence listing, and A and B together preferably represent a contiguous sequence corresponding to the sequence listing entry. Similar fragments from other *hedgehog* also

contemplated, e.g., fragments which correspond to the preferred fragments from the sequence listing entries which are enumerated above.

Isolated peptidyl portions of *hedgehog* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a *hedgehog* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as agonists of a wild-type (e.g., "authentic") *hedgehog* protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

The recombinant *hedgehog* polypeptides of the present invention also include homologs of the authentic *hedgehog* proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. *Hedgehog* homologs of the present invention also include proteins which have been post-translationally modified in a manner different than the authentic protein. Exemplary derivatives of *hedgehog* proteins include polypeptides which lack glycosylation sites (e.g. to produce an unglycosylated protein), which lack sites for cholesterolization, and/or which lack N-terminal and/or C-terminal sequences.

Modification of the structure of the subject *hedgehog* polypeptides can also be for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *hedgehog* polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

It is well known in the art that certain isolated replacements of amino acids, e.g., replacement of an amino acid residue with another related amino acid (i.e. isosteric and/or isoelectric mutations), can be carried out without major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino

acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur-containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *hedgehog* homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

It is specifically contemplated that the methods of the present invention can be carried using homologs of naturally occurring *hedgehog* proteins. In one embodiment, the invention contemplates using *hedgehog* polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hedgehog* homologs which can act as neuroprotective agents. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a cognate receptor, such as *patched*, retaining neuroprotective activity. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein. Moreover, manipulation of certain domains of *hedgehog* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that the review article of Gallop et al. (1994) *J Med Chem* 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239

"[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of *hedgehog* variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the *hedgehog* polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) *Virology* 193:653, and Bass et al. (1990) *Proteins: Structure, Function and Genetics* 8:309-314 also describe other exemplary techniques from the art which can be adapted as means for generating mutagenic variants of *hedgehog* polypeptides.

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of *hedgehog* proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughput analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

To illustrate, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hedgehog* homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hedgehog* sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of *hedgehog* sequences therein.

As illustrated in PCT publication WO 95/18856, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial.

In an illustrative embodiment, alignment of exons 1, 2 and a portion of exon 3 encoded sequences (e.g. the N-terminal approximately 221 residues of the mature protein) of each of the *Shh* clones produces a degenerate set of *Shh* polypeptides represented by the general formula:

5 C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-V-A-E-
K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-Y-N-P-D-I-I-F-
K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-X(4)-L-A-I-S-V-M-N-X(5)-
W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-H-H-X(7)-E-E-S-L-H-Y-E-G-R-A-
V-D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-X(9)-L-X(10)-R-L-A-V-E-A-G-F-D-W-V-
10 Y-Y-E-S-K-A-H-I-H-C-S-V-K-A-E-N-S-V-A-A-K-S-G-G-C-F-P-G-S-A-X(11)-
V-X(12)-L-X(13)-X(14)-G-G-X(15)-K-X(16)-V-K-D-L-X(17)-P-G-D-X(18)-V-
L-A-A-D-X(19)-X(20)-G-X(21)-L-X(22)-X(23)-S-D-F-X(24)-X(25)-F-X(26)-D-
R (SEQ ID No: 19),

wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish *Shh* clones, or, to expand the library, each X
15 can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ; Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7)
20 represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Leu, Val, Met, Thr or Ser; Xaa(12) represents His, Phe, Tyr, Ser, Thr, Met or Cys; Xaa(13) represents Gln, Asn, Glu, or Asp; Xaa(14) represents His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp; Xaa(15) represents Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys; Xaa(16) represents Ala, Gly, Cys, Leu, Val
25 or Met; Xaa(17) represents Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or Cys; Xaa(18) represents Arg, Lys, Met or Ile; Xaa(19) represents Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met; Xaa(20) represents Ala, Gly, Cys, Asp, Asn, Glu or Gln; Xaa(21) represents Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln; Xaa(22) represent Leu, Val, Met or Ile; Xaa(23) represents Phe, Tyr, Thr, His or Trp; Xaa(24) represents Ile, Val, Leu or Met; Xaa(25) represents Met, Cys, Ile,
30 Leu, Val, Thr or Ser; Xaa(26) represents Leu, Val, Met, Thr or Ser. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones, can provide a degenerate polypeptide sequence represented by the general formula:

35 C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6)-P-K-X(7)-L-X(8)-P-L-X(9)-
Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-X(15)-X(16)-E-G-

-31-

5 X(17)-X(18)-X(19)-R-X(20)-S-E-R-F-X(21)-X(22)-L-T-P-N-Y-N-P-D-I-I-F-K-
 D-E-E-N-X(23)-G-A-D-R-L-M-T-X(24)-R-C-K-X(25)-X(26)-X(27)-N-X(28)-L-
 A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-L-R-V-T-E-G-X(31)-D-E-D-G-H-H-
 X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-A-X(35)-D-I-T-T-S-D-R-D-X(36)-X(37)-
 K-Y-G-X(38)-L-X(39)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-X(40)-X(41)-H-
 X(42)-H-X(43)-S-V-K-X(44)-X(45) (SEQIDNo:20),

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a corresponding position in one of the wild-type clones, and may also include amino acid residue which would be conservative substitutions, or each X can be any amino acid residue. In an exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2) represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg; Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6) represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser, Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13) represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys; Xaa(16) represents Gly, Ala, Val, Leu, Ile, Phe or Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21) represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr; Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(29) represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys; Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

There are many ways by which the library of potential *hedgehog* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then

ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hedgehog* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton. Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis techniques.

In one embodiment, the combinatorial library is designed to be secreted (e.g. the polypeptides of the library all include a signal sequence but no transmembrane or cytoplasmic domains), and is used to transfect a eukaryotic cell that can be co-cultured with neuronal cells. A functional *hedgehog* protein secreted by the cells expressing the combinatorial library will diffuse to neighboring neuronal cells and induce a particular biological response, such as protection against cell death under oxygen-deprivation conditions (e.g., high CO₂ culture conditions). The pattern of detection of proliferation will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing *hedgehog* homologs active as neuroprotective agents with respect to neuronal cells

To illustrate, target neuronal cells are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial *hedgehog* gene library and cultured in cell

culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant *hedgehog* homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a
5 period of time sufficient for functional forms of a *hedgehog* protein to produce a measurable response in the target cells, such as neuroprotection, the inserts are removed and the effect of the variant *hedgehog* proteins on the target cells determined. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

10 In yet another screening assay, the candidate *hedgehog* gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as the *patched* protein or other *hedgehog* receptor) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the
15 gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, fluorescently labeled molecules which bind *hedgehog* can be used to score for potentially functional *hedgehog* homologs. Cells can be visually inspected and separated under a fluorescence microscope, or, where the
20 morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant
25 benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E.coli* filamentous phages M13, fd, and f1 are most often used in phage display
libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins
30 without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening *hedgehog* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *hedgehog* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TGI cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hedgehog* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hedgehog*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins which are capable of binding an *hedgehog* receptor are selected or enriched by panning. For instance, the phage library can be applied to cells which express the *patched* protein and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for *hedgehog* homologs, which can then be screened for further biological activities in order to differentiate agonists and antagonists.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, *PNAS USA* 89:7811-7815; Yourvan et al., 1992, *Parallel Problem Solving from Nature*, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

The invention also provides for reduction of the *hedgehog* protein to generate mimetics, e.g. peptide or non-peptide agents, which are able to mimic the neuroprotective activity of a naturally-occurring *hedgehog* polypeptide. Thus, such mutagenic techniques as described above are also useful to map the determinants of the *hedgehog* proteins which participate in protein-

protein interactions involved in, for example, binding of the subject *hedgehog* polypeptide to other extracellular matrix components such as its receptor(s). To illustrate, the critical residues of a subject *hedgehog* polypeptide which are involved in molecular recognition of an *hedgehog* receptor such as *patched* can be determined and used to generate *hedgehog*-derived peptidomimetics which competitively bind with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject *hedgehog* proteins which are involved in binding other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the *hedgehog* protein which facilitate the interaction. After distinguishing between agonist and antagonists, such agonistic mimetics may be used to mimic the normal function of a *hedgehog* protein in the treatment ischemia. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Recombinantly produced forms of the *hedgehog* proteins can be produced using, e.g., expression vectors containing a nucleic acid encoding a *hedgehog* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of a *hedgehog* polypeptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding *hedgehog* polypeptide. Such useful expression control sequences, include, for example, a viral LTR, such

as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In addition to providing a ready source of *hedgehog* polypeptides for purification, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either a neuroprotective form of a *hedgehog* polypeptide. Thus, another aspect of the invention features expression vectors for *in vivo* transfection of a *hedgehog* polypeptide in particular cell types so as cause ectopic expression of a *hedgehog* polypeptide in neuronal tissue.

Formulations of such expression constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the *hedgehog* coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO_4 precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *hedgehog* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular form of the *hedgehog* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding a *hedgehog* polypeptide and renders the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including neuronal cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl.*

Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *hedgehog* gene of the retroviral vector.

Another viral gene delivery system useful in the present method utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including neuronal cells (Rosenfeld et al. (1992) cited *supra*).

Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that

can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7, pp. 109-127). Expression of the inserted *hedgehog* gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a *hedgehog* polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the *hedgehog* polypeptide gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic *hedgehog* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A *hedgehog* expression construct can be delivered in a gene therapy construct to dermal cells by, e.g., electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which

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the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In yet another embodiment, the hedgehog or ptc therapeutic can be a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous gene. For instance, the gene activation construct can replace the endogenous promoter of a *hedgehog* gene with a heterologous promoter, e.g., one which causes constitutive expression of the *hedgehog* gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. Other genes in the *patched* signaling pathway can be similarly targeted. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous *hedgehog* gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic *hedgehog* gene upon recombination of the gene activation construct. For use in generating cultures of *hedgehog* producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native *hedgehog* gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous *hedgehog* gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

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As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In an exemplary embodiment, portions of the 5' flanking region of the human Shh gene are amplified using primers which add restriction sites, to generate the following fragments

5'-gcgcgccttcgaaGCGAGGCAGCCAGCGAGGGAGAGAGCGAGCGGGCGAGCCGGAGC-GAGGAAatcgatgcgcgc (primer 1)

25 5'-gcgcgcagatctGGGAAAGCGCAAGAGAGAGCGCACACGCACACACCCGCCGCGCG-CACTCGgatccgcgcgc (primer 2)

As illustrated, primer 1 includes a 5' non-coding region of the human Shh gene and is flanked by an *Asu*II and *Cl*aI restriction sites. Primer 2 includes a portion of the 5' non-coding region immediately 3' to that present in primer 1. The hedgehog gene sequence is flanked by *Xho*II and *Bam*HI restriction sites. The purified amplimers are cut with each of the enzymes as appropriate.

The vector pCDNA1.1 (Invitrogen) includes a CMV promoter. The plasmid is cut with *Asu*II, which cleaves just 3' to the CMV promoter sequence. The *Asu*II/*Cl*aI fragment of

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primer 1 is ligated to the AsuII cleavage site of the pcDNA vector. The ClaI/AsuII ligation destroys the AsuII site at the 3' end of a properly inserted primer 1.

The vector is then cut with BamHI, and an XhoII/BamHI fragment of primer 2 is ligated to the BamHI cleavage site. As above, the BamHI/XhoII ligation destroys the BamHI site at the 5' end of a properly inserted primer 2.

Individual colonies are selected, cut with AsuII and BamHI, and the size of the AsuII/BamHI fragment determined. Colonies in which both the primer 1 and primer 2 sequences are correctly inserted are further amplified, an cut with AsuII and BamHI to produce the gene activation construct

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10  cgaagcgagggcagccagcgagggagagagcgagcgggagccgagcgaggaaATCGAAGGTT
    CGAATCCTTCCCCCACCACCATCACTTTCAAAGTCCGAAAGAATCTGCTCCCTGCTTGTGTGT
    TGGAGGTCGCTGAGTAGTGCGCGAGTAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAA
    TTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATAT
    ACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATA
15  GCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAA
    CGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTC
    CATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATC
    ATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCA
    GTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACC
20  ATGGTGATGCGGTTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCC
    CAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTGTTTGGCACCAAATCAACGGGACTTTCC
    AAAATGTCGTAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTC
    TATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATA
    CGACTCACTATAGGGAGACCCAAGCTTGGTACCGAGCTCGGATCgatctgggaaagcgcaagag
25  agagcgcacacgcacacacccgcccgcgcactcgg

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In this construct, the flanking primer 1 and primer 2 sequences provide the recombination region which permits the insertion of the CMV promoter in front of the coding sequence for the human *Shh* gene. Other heterologous promoters (or other transcriptional regulatory sequences) can be inserted in a genomic *hedgehog* gene by a similar method.

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression, or ablates a positive control element, e.g., to inhibit expression of the targeted gene.

V. Exemplary ptc therapeutic compounds.

In another embodiment, the subject method is carried out using a *ptc* therapeutic composition. Such compositions can be generated with, for example, compounds which bind to patched and alter its signal transduction activity, compounds which alter the binding and/or enzymatic activity of a protein (e.g., intracellular) involved in patched signal pathway, and compounds which alter the level of expression of a *hedgehog* protein, a patched protein or a protein involved in the intracellular signal transduction pathway of patched.

The availability of purified and recombinant *hedgehog* polypeptides facilitates the generation of assay systems which can be used to screen for drugs, such as small organic molecules, which are either agonists or antagonists of the normal cellular function of a *hedgehog* and/or patched protein, particularly in their role in the pathogenesis of neuronal cell death. In one embodiment, the assay evaluates the ability of a compound to modulate binding between a *hedgehog* polypeptide and a *hedgehog* receptor such as *patched*. In other embodiments, the assay merely scores for the ability of a test compound to alter the signal transduction activity of the *patched* protein. In this manner, a variety of *hedgehog* and/or *ptc* therapeutics, which will include ones with neuroprotective activity, can be identified. A variety of assay formats will suffice and, in light of the present disclosure, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with receptor proteins.

Accordingly, in an exemplary screening assay for *ptc* therapeutics, the compound of interest is contacted with a mixture including a *hedgehog* receptor protein (e.g., a cell expressing the *patched* receptor) and a *hedgehog* protein under conditions in which it is ordinarily capable of binding the *hedgehog* protein. To the mixture is then added a composition containing a test compound. Detection and quantification of receptor/*hedgehog* complexes provides a means for determining the test compound's efficacy at inhibiting (or potentiating) complex formation

between the receptor protein and the *hedgehog* polypeptide. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *hedgehog* polypeptide is added to the receptor protein, and the formation of receptor/*hedgehog* complex is quantitated in the absence of the test compound.

- 5 Agonist and antagonists of neuroprotection can be distinguished, and the efficacy of the compound can be assessed, by subsequent testing with neuronal cells.

 In an illustrative embodiment, the polypeptide utilized as a *hedgehog* receptor can be generated from the *patched* protein. Accordingly, an exemplary screening assay includes all or a suitable portion of the *patched* protein which can be obtained from, for example, the human
10 *patched* gene (GenBank U43148) or other vertebrate sources (see GenBank Accession numbers U40074 for chicken *patched* and U46155 for mouse *patched*), as well as from drosophila (GenBank Accession number M28999) or other invertebrate sources. The *patched* protein can be provided in the screening assay as a whole protein (preferably expressed on the surface of a cell), or alternatively as a fragment of the full length protein which binds to *hedgehog*
15 polypeptides, e.g., as one or both of the substantial extracellular domains (e.g. corresponding to residues Asn120-Ser438 and/or Arg770-Trp1027 of the human *patched* protein). For instance, the *patched* protein can be provided in soluble form, as for example a preparation of one of the extracellular domains, or a preparation of both of the extracellular domains which are covalently
20 connected by an unstructured linker (see, for example, Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513). In other embodiments, the protein can be provided as part of a liposomal preparation or expressed on the surface of a cell. The *patched* protein can derived from a recombinant gene, e.g., being ectopically expressed in a heterologous cell. For instance, the protein can be expressed on oocytes, mammalian cells (e.g., COS, CHO, 3T3 or the like), or yeast cell by standard recombinant DNA techniques. These recombinant cells can be used for
25 receptor binding, signal transduction or gene expression assays. Marigo et al. (1996) *Development* 122:1225-1233 illustrates a binding assay of human *hedgehog* to chick *patched* protein ectopically expressed in *Xenopus laevis* oocytes. The assay system of Marigo et al. can be adapted to the present drug screening assays. As illustrated in that reference, *Shh* binds to the *patched* protein in a selective, saturable, dose-dependent manner, thus demonstrating that
30 *patched* is a receptor for *Shh*.

 Complex formation between the *hedgehog* polypeptide and a *hedgehog* receptor may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labelled proteins such as radiolabelled,

fluorescently labelled, or enzymatically labelled *hedgehog* polypeptides, by immunoassay, or by chromatographic detection.

Typically, for cell-free assays, it will be desirable to immobilize either the *hedgehog* receptor or the *hedgehog* polypeptide to facilitate separation of receptor/*hedgehog* complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/receptor (GST/receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the *hedgehog* polypeptide, e.g. an ^{35}S -labeled *hedgehog* polypeptide, and the test compound and incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound *hedgehog* polypeptide, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the receptor/*hedgehog* complexes are dissociated. Alternatively, the complexes can be dissociated from the bead, separated by SDS-PAGE gel, and the level of *hedgehog* polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, soluble portions of the *hedgehog* receptor protein can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated receptor molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the *hedgehog* receptor but which do not interfere with *hedgehog* binding can be derivatized to the wells of the plate, and the receptor trapped in the wells by antibody conjugation. As above, preparations of a *hedgehog* polypeptide and a test compound are incubated in the receptor-presenting wells of the plate, and the amount of receptor/*hedgehog* complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *hedgehog* polypeptide, or which are reactive with the receptor protein and compete for binding with the *hedgehog* polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *hedgehog* polypeptide. In the instance of the latter, the enzyme can be chemically conjugated or provided

as a fusion protein with the *hedgehog* polypeptide. To illustrate, the *hedgehog* polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of *hedgehog* polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. Likewise, a fusion protein comprising the *hedgehog* polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as the anti-*hedgehog* antibodies described herein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *hedgehog* polypeptide or *hedgehog* receptor sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Where the desired portion of the *hedgehog* receptor (or other *hedgehog* binding molecule) cannot be provided in soluble form, liposomal vesicles can be used to provide manipulatable and isolatable sources of the receptor. For example, both authentic and recombinant forms of the *patched* protein can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374).

In addition to cell-free assays, such as described above, the readily available source of *hedgehog* proteins provided by the art also facilitates the generation of cell-based assays for identifying small molecule agonists of the neuroprotective activity of wild-type *hedgehog* proteins. Analogous to the cell-based assays described above for screening combinatorial libraries, neuronal cells which are sensitive to *hedgehog*-dependent protection against ischemic damage can be contacted with a *hedgehog* protein and a test agent of interest, with the assay scoring for anything from simple binding to the cell to modulation in *hedgehog* inductive responses by the target cell in the presence and absence of the test agent. As with the cell-free

assays. agents which produce a statistically significant change in *hedgehog* activities (either inhibition or potentiation) can be identified.

In addition to characterizing cells that naturally express the *patched* protein, cells which have been genetically engineered to ectopically express *patched* can be utilized for drug screening assays. As an example, cells which either express low levels or lack expression of the *patched* protein, e.g. *Xenopus laevis* oocytes, COS cells or yeast cells, can be genetically modified using standard techniques to ectopically express the *patched* protein. (see Marigo et al., *supra*).

The resulting recombinant cells, e.g., which express a functional *patched* receptor, can be utilized in receptor binding assays to identify agonist or antagonists of *hedgehog* binding. Binding assays can be performed using whole cells. Furthermore, the recombinant cells of the present invention can be engineered to include other heterologous genes encoding proteins involved in *hedgehog*-dependent signal pathways. For example, the gene products of one or more of *smoothed*, *costal-2* and/or *fused* can be co-expressed with *patched* in the reagent cell. with assays being sensitive to the functional reconstitution of the *hedgehog* signal transduction cascade.

Alternatively, liposomal preparations using reconstituted *patched* protein can be utilized. *Patched* protein purified from detergent extracts from both authentic and recombinant origins can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374). The lamellar structure and size of the resulting liposomes can be characterized using electron microscopy. External orientation of the *patched* protein in the reconstituted membranes can be demonstrated, for example, by immunoelectron microscopy. The *hedgehog* protein binding activity of liposomes containing *patched* and liposomes without the protein in the presence of candidate agents can be compared in order to identify potential modulators of the *hedgehog-patched* interaction.

The *hedgehog* protein used in these cell-based assays can be provided as a purified source (natural or recombinant in origin), or in the form of cells/tissue which express the protein and which are co-cultured with the target cells. As in the cell-free assays, where simple binding (rather than induction) is the *hedgehog* activity scored for in the assay, the protein can be

labelled by any of the above-mentioned techniques, e.g., fluorescently, enzymatically or radioactively, or detected by immunoassay.

In addition to binding studies, functional assays can be used to identify modulators, i.e., agonists of *hedgehog* or *patched* activities. By detecting changes in intracellular signals, such as alterations in second messengers or gene expression in *patched*-expressing cells contacted with a test agent, candidate antagonists to *patched* signaling can be identified (e.g., having a *hedgehog*-like activity).

A number of gene products have been implicated in *patched*-mediated signal transduction, including *patched*, the transcription factor *cubitus interruptus* (ci), the serine/threonine kinase *fused* (fu) and the gene products of *costal-2*, *smoothed* and *suppressor of fused*.

The interaction of a *hedgehog* protein with *patched* sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Potential transcriptional targets of *patched* signaling are the *patched* gene itself (Hidalgo and Ingham, 1990 *Development* 110, 291-301; Marigo et al., 1996) and the vertebrate homologs of the drosophila cubitus interruptus gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) *PNAS*, in press; Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). By selecting transcriptional regulatory sequences from such target genes, e.g. from *patched* or *GLI* genes, that are responsible for the up- or down regulation of these genes in response to *patched* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is sensitive to the ability of a specific test compound to modify *patched* signalling pathways. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as antagonists of *ptc*, e.g., which may be useful as neuroprotective agents.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* signaling. To identify potential regulatory elements responsive to *ptc* signaling present in the transcriptional regulatory sequence of a target gene, nested deletions of genomic clones of the target gene can be constructed using standard techniques. See, for example, Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989); U.S. Patent 5,266,488; Sato et al. (1995) *J Biol Chem* 270:10314-10322; and Kube et al. (1995) *Cytokine* 7:1-7. A nested set of DNA fragments from the gene's 5'-flanking region are placed upstream of a reporter gene, such as the luciferase gene, and assayed for their ability to direct reporter gene expression in *patched* expressing cells. Host cells transiently transfected with reporter gene constructs can be scored for the induction of expression of the reporter gene in the presence and absence of *hedgehog* to determine regulatory sequences which are responsive to *patched*-dependent signalling.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers generated by induction with *hedgehog* protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the *hedgehog* activity, with the level of expression of the reporter gene providing the *hedgehog*-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound (or *hedgehog*) or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the signal transduction of the *patched* protein, e.g., the test compound is a potential *ptc* therapeutic.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the

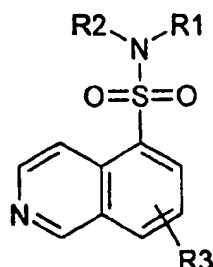
reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is induced after modulation of a *patched* signal transduction pathway. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular stimulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

In yet other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium, phospholipid metabolism or adenylate cyclase activity are quantitated, for instance, the products of phospholipid hydrolysis IP₃, DAG or cAMP could be measured. For example, recent studies have implicated protein kinase A (PKA) as a possible component of *hedgehog/patched* signaling (Hammerschmidt et al. (1996) *Genes & Dev* 10:647). High PKA activity has been shown to antagonize *hedgehog* signaling in these systems. Conversely, inhibitors of PKA will mimic and/or potentiate the action of *hedgehog*. Although it is unclear whether PKA acts directly downstream or in parallel with *hedgehog* signaling, it is possible that *hedgehog* signalling occurs via inhibition of PKA activity. Thus, detection of PKA activity provides a potential readout for the instant assays.

In a preferred embodiment, the *ptc* therapeutic is a PKA inhibitor. A variety of PKA inhibitors are known in the art, including both peptidyl and organic compounds. For instance, the *ptc* therapeutic can be a 5-isoquinolinesulfonamide, such as represented in the general formula:



5

wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or R_8 , or

R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted); R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

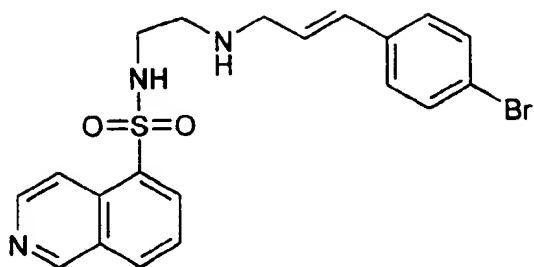
R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

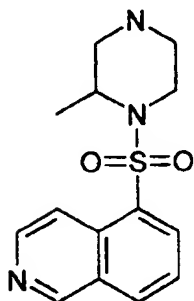
In a preferred embodiment, the PKA inhibitor is N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem Cat. No. 371963), e.g., having the formula:

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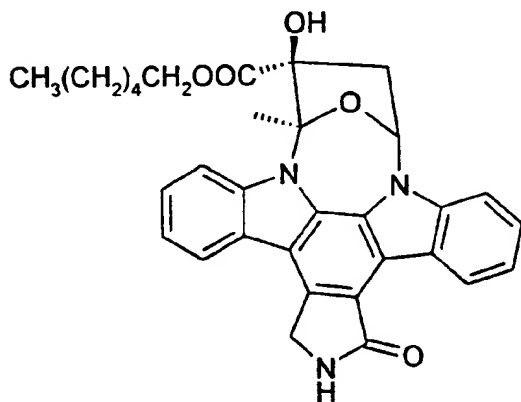
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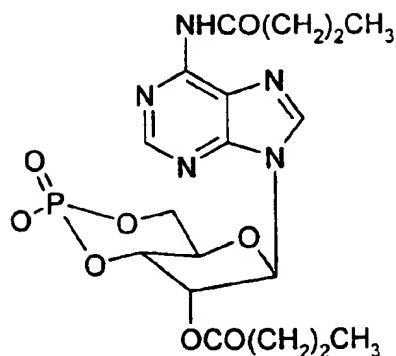
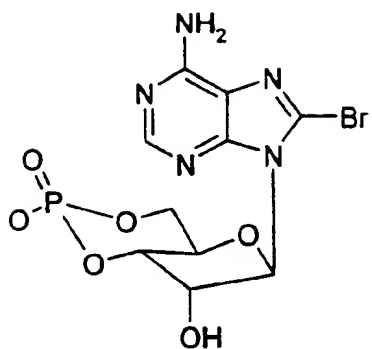
In another embodiment, the PKA inhibitor is 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7; Calbiochem Cat. No. 371955), e.g., having the formula:



- 5 In still other embodiments, the PKA inhibitor is KT5720 (Calbiochem Cat. No. 420315), having the structure



- A variety of nucleoside analogs are also useful as PKA inhibitors. For example, the subject method can be carried out cyclic AMP analogs which inhibit the kinase activity of PKA, as for example, 8-bromo-cAMP or dibutyryl-cAMP
- 10



Exemplary peptidyl inhibitors of PKA activity include the PKA Heat Stable Inhibitor (isoform α : see, for example, Calbiochem Cat. No. 539488, and Wen et al. (1995) *J Biol Chem* 270:2041).

5 Certain *hedgehog* receptors may stimulate the activity of phospholipases. Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. Water soluble derivatives of all three inositol lipids (IP₁, IP₂, IP₃) can also be quantitated using radiolabelling techniques or HPLC.

10 The mobilization of intracellular calcium or the influx of calcium from outside the cell may be a response to *hedgehog* stimulation or lack thereof. Calcium flux in the reagent cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca⁺⁺-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca⁺⁺ detection, cells could be loaded

15 with the Ca⁺⁺-sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca⁺⁺ measured using a fluorometer.

In certain embodiments of the assay, it may be desirable to screen for changes in cellular phosphorylation. As an example, the drosophila gene *fused* (fu) which encodes a serine/threonine kinase has been identified as a potential downstream target in *hedgehog*

20 signaling. (Preat et al., 1990 *Nature* 347, 87-89; Therond et al. 1993, *Mech. Dev.* 44, 65-80). The ability of compounds to modulate serine/threonine kinase activation could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using antibodies against phosphorylated serine or threonine residues. Reagents for performing such assays are commercially available, for example, phosphoserine and phosphothreonine

specific antibodies which measure increases in phosphorylation of those residues can be purchased from commercial sources.

In yet another embodiment, the *ptc* therapeutic is an antisense molecule which inhibits expression of a protein involved in a *patched*-mediated signal transduction pathway. To illustrate, by inhibiting the expression of a protein involved in *patched* signals, such as *fused*, *costal-2*, *smoothened* and/or *Gli* genes, or *patched* itself, the ability of the *patched* signal pathway(s) to alter the ability of a cell to withstand ischemic conditions can be altered, e.g., potentiated or repressed.

As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions with cellular mRNA and/or genomic DNA encoding a *hedgehog* protein, *patched*, or a protein involved in *patched*-mediated signal transduction. The hybridization should inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the target cellular mRNA. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a target gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

Several considerations should be taken into account when constructing antisense oligonucleotides for the use in the methods of the invention: (1) oligos should have a GC content of 50% or more; (2) avoid sequences with stretches of 3 or more G's; and (3) oligonucleotides

should not be longer than 25-26 mers. When testing an antisense oligonucleotide, a mismatched control can be constructed. The controls can be generated by reversing the sequence order of the corresponding antisense oligonucleotide in order to conserve the same ratio of bases.

In an illustrative embodiment, the *ptc* therapeutic can be an antisense construct for inhibiting the expression of *patched*, e.g., to mimic the inhibition of *patched* by *hedgehog*. Exemplary antisense constructs include:

5'-GTCCTGGCGCCGCCGCCGCCGTCGCC

5'-TTCCGATGACCGGCCTTTCGCGGTGA

5'-GTGCACGGAAAGGTGCAGGCCACACT

10

VI. Exemplary pharmaceutical preparations of hedgehog and ptc therapeutics

The source of the *hedgehog* and *ptc* therapeutics to be formulated will depend on the particular form of the agent. Small organic molecules and peptidyl fragments can be chemically synthesized and provided in a pure form suitable for pharmaceutical/cosmetic usage. Products of natural extracts can be purified according to techniques known in the art. For example, the Cox et al. U.S. Patent 5,286,654 describes a method for purifying naturally occurring forms of a secreted protein and can be adapted for purification of *hedgehog* polypeptides. Recombinant sources of *hedgehog* polypeptides are also available. For example, the gene encoding *hedgehog* polypeptides are known, *inter alia*, from PCT publications WO 95/18856 and WO 96/17924.

Those of skill in treating neural tissues can determine the effective amount of an *hedgehog* or *ptc* therapeutic to be formulated in a pharmaceutical or cosmetic preparation.

The *hedgehog* or *ptc* therapeutic formulations used in the method of the invention are most preferably applied in the form of appropriate compositions. As appropriate compositions there may be cited all compositions usually employed for systemically or locally (such as intrathecal) administering drugs. The pharmaceutically acceptable carrier should be substantially inert, so as not to act with the active component. Suitable inert carriers include water, alcohol polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the particular *hedgehog* or *ptc* therapeutic as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical

compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discrete units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications which can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations contain, besides the *hedgehog* or ptc therapeutic, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols,

and the like. If desired, further ingredients may be incorporated in the compositions, e.g. antiinflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrochloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the *hedgehog* or ptc therapeutic, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small (< 2%) amounts of preservative, coloring

agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts (< 2%) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the
5 aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the *hedgehog* or ptc therapeutic is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable amphiphatic
10 molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-
15 soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a *hedgehog* polypeptide, are encapsulated in the aqueous spaces between the molecular layers. The lipid
20 soluble active ingredient of *hedgehog* or ptc therapeutic, such as an organic mimetic, is predominantly incorporated into the lipid layers, although polar head groups may protude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic solvent. When this film is
25 dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-
30 soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid,

liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of *hedgehog* and ptc therapeutics is the method described in EP-A-253,619, incorporated herein by reference.

5 In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

10 The single bilayered liposomes containing the encapsulated *hedgehog* or ptc therapeutic can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for localized administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous
15 component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous
20 component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are formed in the mixing means and then transferred to
25 another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, phosphatidylserine, phosphatidylethanol-
30 amine, phosphatidylinositol, lysophosphatidylcholine and phosphatidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such as benzoic acid, methyl paraben and propyl paraben may also be added.

5 Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of an *hh* at a particular target
10 site. Such embodiments of the present invention can be used for the delivery of an exogenously purified *hedgehog* protein, which has been incorporated in the polymeric device, or for the delivery of *hedgehog* produced by a cell encapsulated in the polymeric device.

 An essential feature of certain embodiments of the implant can be the linear release of the therapeutic, which can be achieved through the manipulation of the polymer composition and
15 form. By choice of monomer composition or polymerization technique, the amount of water, porosity and consequent permeability characteristics can be controlled. The selection of the shape, size, polymer, and method for implantation can be determined on an individual basis according to the disorder to be treated and the individual patient response. The generation of such implants is generally known in the art. See, for example, *Concise Encyclopedia of Medical*
20 *& Dental Materials*, ed. by David Williams (MIT Press: Cambridge, MA, 1990); and the Sabel et al. U.S. Patent No. 4,883,666.

 In another embodiment of an implant, a source of cells producing the therapeutic, e.g., secreting a soluble form of a *hedgehog* protein, is encapsulated in implantable hollow fibers or the like. Such fibers can be pre-spun and subsequently loaded with the cell source (Aebischer et
25 al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) *Expt. Neurobiol.* 110:39-44; Jaeger et al. (1990) *Prog. Brain Res.* 82:41-46; and Aebischer et al. (1991) *J. Biomech. Eng.* 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) *Trans. Am. Artif. Intern. Organs* 35:791-799; Sefton et al.
30 (1987) *Biotechnol. Bioeng.* 29:1135-1143; and Aebischer et al. (1991) *Biomaterials* 12:50-55).

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Sonic Hedgehog (Shh) was evaluated in the focal stroke model involving permanent middle cerebral artery occlusion (MCAO) in the spontaneously hypertensive rat. Samples of the proteins were tested as a neuroprotective agent by measuring the volume of cerebral infarction, by means of vital dye exclusion, in animals receiving systemic injections. For review of the MCAO, see Tamura et al. (1981) *J Cerebral Blood Flow and Metabolism* 1:53-60.

Briefly, male Wistar rats, weighing about 270-300g were treated systemically with *Shh* at 500 µg/kg/hr for 3 hrs at 0.5 ml/hr. Control animals received buffer at same dilution as *Shh* stock for the same period of time and volumes.

Prior to administration of the *Shh* or control stocks, the MCAO animals were generated as follows: the rats were anesthetized, with 400 mg/ml chloral hydrate, and their femoral vein and artery were cannulated. Mean arterial blood pressure was monitored and blood samples taken for blood gas measurements. A half-hour later, the middle cerebral artery was occluded with a nylon monofilament suture inserted via carotid artery. Half-hour after onset of occlusion, having allowed animal to awake, infusion of *Shh* or buffer/vehicle was started. The catheters were removed, and the animals were returned to their cages. At 24 hours post-surgery, the animals sacrificed by decapitation. Their brains were removed and cut into 2 mm serial, coronal sections. The sections stained with TTC stain and then fixed in neutral buffered formalin. Infarct volumes measured by quantitative morphometry and expressed as a percentage of the total hemispheric volume (normalized against the contralateral hemisphere to correct for edema-associated swelling).

Figure 1 illustrates the results of the above-referenced experiments. A substantial decrease in the volume of the cerebral infarct was observed in the *hedgehog* treated rats relative to the control rats. While not shown in Figure 1, it was further observed that there was no statistically significant effect of *hedgehog* on blood pressure, pH, pO₂, or pCO₂.

All of the above-cited references and publications are hereby incorporated by reference.

SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT: ONTOGENY, INC.

(A) STREET: 45 Moulton Street

(B) CITY: Cambridge

10 (C) STATE: Massachusetts

(D) COUNTRY: United States of America

(E) ZIP: 02138

(ii) TITLE OF INVENTION: NEUROPROTECTIVE METHODS AND REAGENTS

15

(iii) NUMBER OF SEQUENCES: 26

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: FOLEY, HOAG & ELIOT LLP

20 (B) STREET: One Post Office Square

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(D) STATE: MA

(E) COUNTRY: USA

(F) ZIP: 02109-2170

25

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

30 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/883,656

(B) FILING DATE: 27-JUN-1997

35 (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Vincent, Matthew P.

(B) REGISTRATION NUMBER: 36,709

40 (C) REFERENCE/DOCKET NUMBER: ONV-043.01

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-832-1000

-63-

(B) TELEFAX: 617-832-7000

5 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1277 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1275

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile	
1 5 10 15	
TGC GGT CTT TTA GTC TCC TCT GGG CTG ACT TGT GGA CCA GGC AGG GGC	96
Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly	
20 25 30	
ATT GGA AAA AGG AGG CAC CCC AAA AAG CTG ACC CCG TTA GCC TAT AAG	144
Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys	
35 40 45	
CAG TTT ATT CCC AAT GTG GCA GAG AAG ACC CTA GGG GCC AGT GGA AGA	192
Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg	
50 55 60	
TAT GAA GGG AAG ATC ACA AGA AAC TCC GAG AGA TTT AAA GAA CTA ACC	240
Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr	
65 70 75 80	
CCA AAT TAC AAC CCT GAC ATT ATT TTT AAG GAT GAA GAG AAC ACG GGA	288
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly	
85 90 95	
GCT GAC AGA CTG ATG ACT CAG CGC TGC AAG GAC AAG CTG AAT GCC CTG	336
Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu	
100 105 110	
GCG ATC TCG GTG ATG AAC CAG TGG CCC GGG GTG AAG CTG CGG GTG ACC	384
Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr	
115 120 125	
GAG GGC TGG GAC GAG GAT GGC CAT CAC TCC GAG GAA TCG CTG CAC TAC	432
Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr	
130 135 140	
GAG GGT CGC GCC GTG GAC ATC ACC ACG TCG GAT CGG GAC CGC AGC AAG	480
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys	
145 150 155 160	
TAC GGA ATG CTG GCC CGC CTC GCC GTC GAG GCC GGC TTC GAC TGG GTC	528
Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val	
165 170 175	
TAC TAC GAG TCC AAG GCG CAC ATC CAC TGC TCC GTC AAA GCA GAA AAC	576
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn	
180 185 190	

-64-

	TCA GTG GCA GCG AAA TCA GGA GGC TGC TTC CCT GGC TCA GCC ACA GTG	624	-
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	195 200 205		
5	CAC CTG GAG CAT GGA GGC ACC AAG CTG GTG AAG GAC CTG AGC CCT GGG	672	
	His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly		
	210 215 220		
10	GAC CGC GTG CTG GCT GCT GAC GCG GAC GGC CGG CTG CTC TAC AGT GAC	720	
	Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp		
	225 230 235 240		
15	TTC CTC ACC TTC CTC GAC CGG ATG GAC AGC TCC CGA AAG CTC TTC TAC	768	
	Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr		
	245 250 255		
20	GTC ATC GAG ACG CGG CAG CCC CGG GCC CGG CTG CTA CTG ACG GCG GCC	816	
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	260 265 270		
	CAC CTG CTC TTT GTG GCC CCC CAG CAC AAC CAG TCG GAG GCC ACA GGG	864	
	His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly		
	275 280 285		
25	TCC ACC AGT GGC CAG GCG CTC TTC GCC AGC AAC GTG AAG CCT GGC CAA	912	
	Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln		
	290 295 300		
30	CGT GTC TAT GTG CTG GGC GAG GGC GGG CAG CAG CTG CTG CCG GCG TCT	960	
	Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser		
	305 310 315 320		
35	GTC CAC AGC GTC TCA TTG CGG GAG GAG GCG TCC GGA GCC TAC GCC CCA	1008	
	Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro		
	325 330 335		
40	CTC ACC GCC CAG GGC ACC ATC CTC ATC AAC CGG GTG TTG GCC TCC TGC	1056	
	Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys		
	340 345 350		
	TAC GCC GTC ATC GAG GAG CAC AGT TGG GCC CAT TGG CCC TTC GCA CCA	1104	
	Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro		
	355 360 365		
45	TTC CGC TTG GCT CAG GGG CTG CTG GCC GCC CTC TGC CCA GAT GGG GCC	1152	
	Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala		
	370 375 380		
50	ATC CCT ACT GCC GCC ACC ACC ACC ACT GGC ATC CAT TGG TAC TCA CGG	1200	
	Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg		
	385 390 395 400		
55	CTC CTC TAC CGC ATC GGC AGC TGG GTG CTG GAT GGT GAC GCG CTG CAT	1248	
	Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His		
	405 410 415		
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	Pro Leu Gly Met Val Ala Pro Ala Ser		
	420 425		

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1190 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

-65-

(ii) MOLECULE TYPE: cDNA

5 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1191

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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	1 5 10 15	
	GCA CTA TCT GCC CAG AGC TGC GGG CCG GGC CGA GGA CCG GTT GGC CGG	96
	Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg	
	20 25 30	
20	CGG CGT TAT GTG CGC AAG CAA CTT GTG CCT CTG CTA TAC AAG CAG TTT	144
	Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe	
	35 40 45	
25	GTG CCC AGT ATG CCC GAG CGG ACC CTG GGC GCG AGT GGG CCA GCG GAG	192
	Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu	
	50 55 60	
30	GGG AGG GTA ACA AGG GGG TCG GAG CGC TTC CGG GAC CTC GTA CCC AAC	240
	Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn	
	65 70 75 80	
	TAC AAC CCC GAC ATA ATC TTC AAG GAT GAG GAG AAC AGC GGC GCA GAC	288
	Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp	
35	85 90 95	
	CGC CTG ATG ACA GAG CGT TGC AAA GAG CGG GTG AAC GCT CTA GCC ATC	336
	Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile	
	100 105 110	
40	GCG GTG ATG AAC ATG TGG CCC GGA GTA CGC CTA CGT GTG ACT GAA GGC	384
	Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly	
	115 120 125	
45	TGG GAC GAG GAC GGC CAC CAC GCA CAG GAT TCA CTC CAC TAC GAA GGC	432
	Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly	
	130 135 140	
50	CGT GCC TTG GAC ATC ACC ACG TCT GAC CGT GAC CGT AAT AAG TAT GGT	480
	Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly	
	145 150 155 160	
	TTG TTG GCG CGC CTA GCT GTG GAA GCC GGA TTC GAC TGG GTC TAC TAC	528
	Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr	
55	165 170 175	
	GAG TCC CGC AAC CAC ATC CAC GTA TCG GTC AAA GCT GAT AAC TCA CTG	576
	Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu	
	180 185 190	
60	GCG GTC CGA GCC GGA GGC TGC TTT CCG GGA AAT GCC ACG GTG CGC TTG	624
	Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu	
	195 200 205	
65	CGG AGC GGC GAA CGG AAG GGG CTG AGG GAA CTA CAT CGT GGT GAC TGG	672
	Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp	
	210 215 220	

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	225					230					235				240		
5	CTC	TTC	CTG	GAC	CGG	GAT	CTG	CAG	CGC	CGC	GCC	TCG	TTC	GTG	GCT	GTG	768
	Leu	Phe	Leu	Asp	Arg	Asp	Leu	Gln	Arg	Arg	Ala	Ser	Phe	Val	Ala	Val	
					245					250					255		
10	GAG	ACC	GAG	CGG	CCT	CCG	CGC	AAA	CTG	TTG	CTC	ACA	CCC	TGG	CAT	CTG	816
	Glu	Thr	Glu	Arg	Pro	Pro	Arg	Lys	Leu	Leu	Leu	Thr	Pro	Trp	His	Leu	
				260					265					270			
15	GTG	TTC	GCT	GCT	CGC	GGG	CCA	GCG	CCT	GCT	CCA	GGT	GAC	TTT	GCA	CCG	864
	Val	Phe	Ala	Ala	Arg	Gly	Pro	Ala	Pro	Ala	Pro	Gly	Asp	Phe	Ala	Pro	
			275					280					285				
20	GTG	TTC	GCG	CGC	CGC	TTA	CGT	GCT	GGC	GAC	TCG	GTG	CTG	GCT	CCC	GGC	912
	Val	Phe	Ala	Arg	Arg	Leu	Arg	Ala	Gly	Asp	Ser	Val	Leu	Ala	Pro	Gly	
			290				295					300					
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	Gly	Asp	Ala	Leu	Gln	Pro	Ala	Arg	Val	Ala	Arg	Val	Ala	Arg	Glu	Glu	
	305					310					315				320		
25	GCC	GTG	GGC	GTG	TTC	GCA	CCG	CTC	ACT	GCG	CAC	GGG	ACG	CTG	CTG	GTC	1008
	Ala	Val	Gly	Val	Phe	Ala	Pro	Leu	Thr	Ala	His	Gly	Thr	Leu	Leu	Val	
					325					330					335		
30	AAC	GAC	GTC	CTC	GCC	TCC	TGC	TAC	GCG	GTT	CTA	GAG	AGT	CAC	CAG	TGG	1056
	Asn	Asp	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Leu	Glu	Ser	His	Gln	Trp	
				340					345					350			
35	GCC	CAC	CGC	GCC	TTC	GCC	CCT	TTG	CGG	CTG	CTG	CAC	GCG	CTC	GGG	GCT	1104
	Ala	His	Arg	Ala	Phe	Ala	Pro	Leu	Arg	Leu	Leu	His	Ala	Leu	Gly	Ala	
			355					360					365				
40	CTG	CTC	CCT	GGG	GGT	GCA	GTC	CAG	CCG	ACT	GGC	ATG	CAT	TGG	TAC	TCT	1152
	Leu	Leu	Pro	Gly	Gly	Ala	Val	Gln	Pro	Thr	Gly	Met	His	Trp	Tyr	Ser	
			370				375					380					
	CGC	CTC	CTT	TAC	CGC	TTG	GCC	GAG	GAG	TTA	ATG	GGC	TG				1190
	Arg	Leu	Leu	Tyr	Arg	Leu	Ala	Glu	Glu	Leu	Met	Gly					
	385					390					395						
45	(2) INFORMATION FOR SEQ ID NO:3:																
	(i) SEQUENCE CHARACTERISTICS:																
50	(A) LENGTH: 1281 base pairs																
	(B) TYPE: nucleic acid																
	(C) STRANDEDNESS: both																
	(D) TOPOLOGY: linear																
55	(ii) MOLECULE TYPE: cDNA																
	(ix) FEATURE:																
60	(A) NAME/KEY: CDS																
	(B) LOCATION: 1..1233																
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:																
65	ATG	TCT	CCC	GCC	TGG	CTC	CGG	CCC	CGA	CTG	CGG	TTC	TGT	CTG	TTC	CTG	48
	Met	Ser	Pro	Ala	Trp	Leu	Arg	Pro	Arg	Leu	Arg	Phe	Cys	Leu	Phe	Leu	
	1				5					10				15			
	CTG	CTG	CTG	CTT	CTG	GTG	CCG	GCG	GCG	CGG	GGC	TGC	GGG	CCG	GGC	CGG	96

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	Leu	Leu	Leu	Leu	Leu	Val	Pro	Ala	Ala	Arg	Gly	Cys	Gly	Pro	Gly	Arg	
				20					25					30			-
5	GTG	GTG	GGC	AGC	CGC	CGG	AGG	CCG	CCT	CGC	AAG	CTC	GTG	CCT	CTT	GCC	144
	Val	Val	Gly	Ser	Arg	Arg	Arg	Pro	Pro	Arg	Lys	Leu	Val	Pro	Leu	Ala	
			35					40				45					
10	TAC	AAG	CAG	TTC	AGC	CCC	AAC	GTG	CCG	GAG	AAG	ACC	CTG	GGC	GCC	AGC	192
	Tyr	Lys	Gln	Phe	Ser	Pro	Asn	Val	Pro	Glu	Lys	Thr	Leu	Gly	Ala	Ser	
		50					55					60					
15	GGG	CGC	TAC	GAA	GGC	AAG	ATC	GCG	CGC	AGC	TCT	GAG	CGC	TTC	AAA	GAG	240
	Gly	Arg	Tyr	Glu	Gly	Lys	Ile	Ala	Arg	Ser	Ser	Glu	Arg	Phe	Lys	Glu	
	65					70				75						80	
20	CTC	ACC	CCC	AAC	TAC	AAT	CCC	GAC	ATC	ATC	TTC	AAG	GAC	GAG	GAG	AAC	288
	Leu	Thr	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	
				85						90					95		
25	ACG	GGT	GCC	GAC	CGC	CTC	ATG	ACC	CAG	CGC	TGC	AAG	GAC	CGT	CTG	AAC	336
	Thr	Gly	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Arg	Leu	Asn	
				100					105					110			
30	TCA	CTG	GCC	ATC	TCT	STC	ATG	AAC	CAG	TGG	CCT	GGT	GTG	AAA	CTG	CGG	384
	Ser	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	
			115					120					125				
35	GTG	ACC	GAA	GGC	CGG	GAT	GAA	GAT	GGC	CAT	CAC	TCA	GAG	GAG	TCT	TTA	432
	Val	Thr	Glu	Gly	Arg	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	
		130					135					140					
40	CAC	TAT	GAG	GGC	CGC	GCG	GTG	GAT	ATC	ACC	ACC	TCA	GAC	CGT	GAC	CGA	480
	His	Tyr	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	
	145					150				155						160	
45	AAT	AAG	TAT	GGA	CTG	CTG	GCG	CGC	TTA	GCA	GTG	GAG	GCC	GGC	TTC	GAC	528
	Asn	Lys	Tyr	Gly	Leu	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	
				165					170						175		
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	Trp	Val	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Val	His	Cys	Ser	Val	Lys	Ser	
				180					185					190			
55	GAG	CAT	TCG	GCC	GCT	GCC	AAG	ACA	GGT	GGC	TGC	TTT	CCT	GCC	GGA	GCC	624
	Glu	His	Ser	Ala	Ala	Ala	Lys	Thr	Gly	Gly	Cys	Phe	Pro	Ala	Gly	Ala	
			195					200					205				
60	CAG	GTG	CGC	CTA	GAG	AAC	GGG	GAG	CGT	GTG	GCC	CTG	TCA	GCT	GTA	AAG	672
	Gln	Val	Arg	Leu	Glu	Asn	Gly	Glu	Arg	Val	Ala	Leu	Ser	Ala	Val	Lys	
		210					215					220					
65	CCA	GGA	GAC	CGG	GTG	CTG	GCC	ATG	GGG	GAG	GAT	GGG	ACC	CCC	ACC	TTC	720
	Pro	Gly	Asp	Arg	Val	Leu	Ala	Met	Gly	Glu	Asp	Gly	Thr	Pro	Thr	Phe	
		225				230				235						240	
70	AGT	GAT	GTG	CTT	ATT	TTC	CTG	GAC	CGC	GAG	CCA	AAC	CGG	CTG	AGA	GCT	768
	Ser	Asp	Val	Leu	Ile	Phe	Leu	Asp	Arg	Glu	Pro	Asn	Arg	Leu	Arg	Ala	
				245					250						255		
75	TTC	CAG	GTC	ATC	GAG	ACT	CAG	GAT	CCT	CCG	CGT	CGG	CTG	GCG	CTC	ACG	816
	Phe	Gln	Val	Ile	Glu	Thr	Gln	Asp	Pro	Pro	Arg	Arg	Leu	Ala	Leu	Thr	
				260					265					270			
80	CCT	GCC	CAC	CTG	CTC	TTC	ATT	GCG	GAC	AAT	CAT	ACA	GAA	CCA	GCA	GCC	864
	Pro	Ala	His	Leu	Leu	Phe	Ile	Ala	Asp	Asn	His	Thr	Glu	Pro	Ala	Ala	
			275					280					285				
85	CAC	TTC	CGG	GCC	ACA	TTT	GCC	AGC	CAT	GTG	CAA	CCA	GGC	CAA	TAT	GTG	912

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	His	Phe	Arg	Ala	Thr	Phe	Ala	Ser	His	Val	Gln	Pro	Gly	Gln	Tyr	Val	
	290						295					300					
5	CTG	GTA	TCA	GGG	GTA	CCA	GGC	CTC	CAG	CCT	GCT	CGG	GTG	GCA	GCT	GTC	960
	Leu	Val	Ser	Gly	Val	Pro	Gly	Leu	Gln	Pro	Ala	Arg	Val	Ala	Ala	Val	
	305					310					315					320	
10	TCC	ACC	CAC	GTG	GCC	CTT	GGG	TCC	TAT	GCT	CCT	CTC	ACA	AGG	CAT	GGG	1008
	Ser	Thr	His	Val	Ala	Leu	Gly	Ser	Tyr	Ala	Pro	Leu	Thr	Arg	His	Gly	
					325					330					335		
15	ACA	CTT	GTG	GTG	GAG	GAT	GTG	GTG	GCC	TCC	TGC	TTT	GCA	GCT	GTG	GCT	1056
	Thr	Leu	Val	Val	Glu	Asp	Val	Val	Ala	Ser	Cys	Phe	Ala	Ala	Val	Ala	
					340				345					350			
20	GAC	CAC	CAT	CTG	GCT	CAG	TTG	GCC	TTC	TGG	CCC	CTG	CGA	CTG	TTT	CCC	1104
	Asp	His	His	Leu	Ala	Gln	Leu	Ala	Phe	Trp	Pro	Leu	Arg	Leu	Phe	Pro	
			355					360					365				
25	AGT	TTG	GCA	TGG	GGC	AGC	TGG	ACC	CCA	AGT	GAG	GGT	GTT	CAC	TCC	TAC	1152
	Ser	Leu	Ala	Trp	Gly	Ser	Trp	Thr	Pro	Ser	Glu	Gly	Val	His	Ser	Tyr	
		370					375					380					
30	CCT	CAG	ATG	CTC	TAC	CGC	CTG	GGG	CGT	CTC	TTG	CTA	GAA	GAG	AGC	ACC	1200
	Pro	Gln	Met	Leu	Tyr	Arg	Leu	Gly	Arg	Leu	Leu	Leu	Glu	Glu	Ser	Thr	
						390					395					400	
35	TTC	CAT	CCA	CTG	GGC	ATG	TCT	GGG	GCA	GGA	AGC	TGAAGGGACT	CTAACCACTG				1253
	Phe	His	Pro	Leu	Gly	Met	Ser	Gly	Ala	Gly	Ser						
					405					410							
40	CCCTCCTGGA	ACTGCTGTGC	GTGGATCC														1281
45	(2)	INFORMATION FOR SEQ ID NO:4:															
50	(i)	SEQUENCE CHARACTERISTICS:															
		(A) LENGTH: 1313 base pairs															
		(B) TYPE: nucleic acid															
		(C) STRANDEDNESS: both															
		(D) TOPOLOGY: linear															
55	(ii)	MOLECULE TYPE: cDNA															
60	(ix)	FEATURE:															
		(A) NAME/KEY: CDS															
		(B) LOCATION: 1..1314															
65	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:4:															
70	ATG	CTG	CTG	CTG	CTG	GCC	AGA	TGT	TTT	CTG	GTG	ATC	CTT	GCT	TCC	TCG	48
	Met	Leu	Leu	Leu	Leu	Ala	Arg	Cys	Phe	Leu	Val	Ile	Leu	Ala	Ser	Ser	
	1					5				10					15		
75	CTG	CTG	GTG	TGC	CCC	GGG	CTG	GCC	TGT	GGG	CCC	GGC	AGG	GGG	TTT	GGA	96
	Leu	Leu	Val	Cys	Pro	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Phe	Gly	
				20					25					30			
80	AAG	AGG	CGG	CAC	CCC	AAA	AAG	CTG	ACC	CCT	TTA	GCC	TAC	AAG	CAG	TTT	144
	Lys	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	
			35					40					45				
85	ATT	CCC	AAC	GTA	GCC	GAG	AAG	ACC	CTA	GGG	GCC	AGC	GGC	AGA	TAT	GAA	192
	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	
		50					55					60					

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	GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA CTC ACC CCC AAT	240
	Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn	
	65 70 75 80	
5	TAC AAC CCC GAC ATC ATA TTT AAG GAT GAG GAA AAC ACG GGA GCA GAC	288
	Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp	
	85 90 95	
10	CGG CTG ATG ACT CAG AGG TGC AAA GAC AAG TTA AAT GCC TTG GCC ATC	336
	Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile	
	100 105 110	
15	TCT GTG ATG AAC CAG TGG CCT GGA GTG AGG CTG CGA GTG ACC GAG GGC	384
	Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly	
	115 120 125	
20	TGG GAT GAG GAC GGC CAT CAT TCA GAG GAG TCT CTA CAC TAT GAG GGT	432
	Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly	
	130 135 140	
25	CGA GCA GTG GAC ATC ACC ACG TCC GAC CGG GAC CGC AGC AAG TAC GGC	480
	Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly	
	145 150 155 160	
30	ATG CTG GCT CGC CTG GCT GTG GAA GCA GGT TTC GAC TGG GTC TAC TAT	528
	Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr	
	165 170 175	
35	GAA TCC AAA GCT CAC ATC CAC TGT TCT GTG AAA GCA GAG AAC TCC GTG	576
	Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val	
	180 185 190	
40	CGC GCC AAA TCC GGC GGC TGT TTC CCG GGA TCC GCC ACC GTG CAC CTG	624
	Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu	
	195 200 205	
45	GAG CAG GGC GGC ACC AAG CTG GTG AAG GAC TTA CGT CCC GGA GAC CGC	672
	Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg	
	210 215 220	
50	GTG CTG GCG GCT GAC GAC CAG GGC CGG CTG CTG TAC AGC GAC TTC CTC	720
	Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu	
	225 230 235 240	
55	ACC TTC CTG GAC CGC GAC GAA GGC GCC AAG AAG GTC TTC TAC GTG ATC	768
	Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile	
	245 250 255	
60	GAG ACG CTG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG	816
	Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu	
	260 265 270	
65	CTC TTC GTG GCG CCG CAC AAC GAC TCG GGG CCC ACG CCC GGG CCA AGC	864
	Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser	
	275 280 285	
70	GCG CTC TTT GCC AGC CGC GTG CGC CCC GGG CAG CGC GTG TAC GTG GTG	912
	Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val	
	290 295 300	
75	GCT GAA CGC GGC GGG GAC CGC CGG CTG CTG CCC GCC GCG GTG CAC AGC	960
	Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser	
	305 310 315 320	
80	GTG ACG CTG CGA GAG GAG GAG GCG GGC GCG TAC GCG CCG CTC ACG GCG	1008
	Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala	
	325 330 335	

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	CAC GGC ACC ATT CTC ATC AAC CGG GTG CTC GCC TCG TGC TAC GCT GTC	1056	
	His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val		-
	340 345 350		
5	ATC GAG GAG CAC AGC TGG GCA CAC CGG GCC TTC GCG CCT TTC CGC CTG	1104	
	Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu		
	355 360 365		
10	GCG CAC GCG CTG CTG GCC GCG CTG GCA CCC GCC CGC ACG GAC GGC GGG	1152	
	Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly		
	370 375 380		
15	GGC GGG GGC AGC ATC CCT GCA GCG CAA TCT GCA ACG GAA GCG AGG GGC	1200	
	Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly		
	385 390 395 400		
20	GCG GAG GCG ACT GCG GGC ATC CAC TGG TAC TCG CAG CTG CTC TAC CAC	1248	
	Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His		
	405 410 415		
25	ATT GGC ACC TGG CTG TTG GAC AGC GAG ACC ATG CAT CCC TTG GGA ATG	1296	
	Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met		
	420 425 430		
30	GCG GTC AAG TCC AGC TG	1313	
	Ala Val Lys Ser Ser		
	435		
35	(2) INFORMATION FOR SEQ ID NO:5:		
	(i) SEQUENCE CHARACTERISTICS:		
	(A) LENGTH: 1256 base pairs		
	(B) TYPE: nucleic acid		
	(C) STRANDEDNESS: both		
	(D) TOPOLOGY: linear		
40	(ii) MOLECULE TYPE: cDNA		
	(ix) FEATURE:		
	(A) NAME/KEY: CDS		
	(B) LOCATION: 1..1257		
45	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:		
50	ATG CGG CTT TTG ACG AGA GTG CTG CTG GTG TCT CTT CTC ACT CTG TCC	48	
	Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser		
	1 5 10 15		
55	TTG GTG GTG TCC GGA CTG GCC TGC GGT CCT GGC AGA GGC TAC GGC AGA	96	
	Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg		
	20 25 30		
60	AGA AGA CAT CCG AAG AAG CTG ACA CCT CTC GCC TAC AAG CAG TTC ATA	144	
	Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile		
	35 40 45		
65	CCT AAT GTC GCG GAG AAG ACC TTA GGG GCC AGC GGC AGA TAC GAG GGC	192	
	Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly		
	50 55 60		
70	AAG ATA ACG CGC AAT TCG GAG AGA TTT AAA GAA CTT ACT CCA AAT TAC	240	
	Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr		
	65 70 75 80		
	AAT CCC GAC ATT ATC TTT AAG GAT GAG GAG AAC ACG GGA GCG GAC AGG	288	

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	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	
					85					90					95		
5	CTC	ATG	ACA	CAG	AGA	TGC	AAA	GAC	AAG	CTG	AAC	TCG	CTG	GCC	ATC	TCT	336
	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu	Ala	Ile	Ser	
				100					105					110			
10	GTA	ATG	AAC	CAC	TGG	CCA	GGG	GTT	AAG	CTG	CGT	GTG	ACA	GAG	GGC	TGG	384
	Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	
			115					120					125				
15	GAT	GAG	GAC	GGT	CAC	CAT	TTT	GAA	GAA	TCA	CTC	CAC	TAC	GAG	GGA	AGA	432
	Asp	Glu	Asp	Gly	His	His	Phe	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	
			130				135					140					
20	GCT	GTT	GAT	ATT	ACC	ACC	TCT	GAC	CGA	GAC	AAG	AGC	AAA	TAC	GGG	ACA	480
	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys	Tyr	Gly	Thr	
						150					155					160	
25	CTG	TCT	CGC	CTA	GCT	GTG	GAG	GCT	GGA	TTT	GAC	TGG	GTC	TAT	TAC	GAG	528
	Leu	Ser	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	
					165					170					175		
30	TCC	AAA	GCC	CAC	ATT	CAT	TGC	TCT	GTC	AAA	GCA	GAA	AAT	TCG	GTT	GCT	576
	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	
				180					185					190			
35	GCG	AAA	TCT	GGG	GGC	TGT	TTC	CCA	GGT	TCG	GCT	CTG	GTC	TCG	CTC	CAG	624
	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Leu	Val	Ser	Leu	Gln	
			195					200					205				
40	GAC	GGA	GGA	CAG	AAG	GCC	GTG	AAG	GAC	CTG	AAC	CCC	GGA	GAC	AAG	GTG	672
	Asp	Gly	Gly	Gln	Lys	Ala	Val	Lys	Asp	Leu	Asn	Pro	Gly	Asp	Lys	Val	
			210				215					220					
45	CTG	GCG	GCA	GAC	AGC	GCG	GGA	AAC	CTG	GTG	TTC	AGC	GAC	TTC	ATC	ATG	720
	Leu	Ala	Ala	Asp	Ser	Ala	Gly	Asn	Leu	Val	Phe	Ser	Asp	Phe	Ile	Met	
						230				235						240	
50	TTC	ACA	GAC	CGA	GAC	TCC	ACG	ACG	CGA	CGT	GTG	TTT	TAC	GTC	ATA	GAA	768
	Phe	Thr	Asp	Arg	Asp	Ser	Thr	Thr	Arg	Arg	Val	Phe	Tyr	Val	Ile	Glu	
					245					250					255		
55	ACG	CAA	GAA	CCC	GTT	GAA	AAG	ATC	ACC	CTC	ACC	GCC	GCT	CAC	CTC	CTT	816
	Thr	Gln	Glu	Pro	Val	Glu	Lys	Ile	Thr	Leu	Thr	Ala	Ala	His	Leu	Leu	
				260				265						270			
60	TTT	GTC	CTC	GAC	AAC	TCA	ACG	GAA	GAT	CTC	CAC	ACC	ATG	ACC	GCC	GCG	864
	Phe	Val	Leu	Asp	Asn	Ser	Thr	Glu	Asp	Leu	His	Thr	Met	Thr	Ala	Ala	
			275					280					285				
65	TAT	GCC	AGC	AGT	GTC	AGA	GCC	GGA	CAA	AAG	GTG	ATG	GTT	GTT	GAT	GAT	912
	Tyr	Ala	Ser	Ser	Val	Arg	Ala	Gly	Gln	Lys	Val	Met	Val	Val	Asp	Asp	
			290				295					300					
70	AGC	GGT	CAG	CTT	AAA	TCT	GTC	ATC	GTG	CAG	CGG	ATA	TAC	ACG	GAG	GAG	960
	Ser	Gly	Gln	Leu	Lys	Ser	Val	Ile	Val	Gln	Arg	Ile	Tyr	Thr	Glu	Glu	
			305				310				315					320	
75	CAG	CGG	GGC	TCG	TTC	GCA	CCA	GTG	ACT	GCA	CAT	GGG	ACC	ATT	GTG	GTC	1008
	Gln	Arg	Gly	Ser	Phe	Ala	Pro	Val	Thr	Ala	His	Gly	Thr	Ile	Val	Val	
					325					330					335		
80	GAC	AGA	ATA	CTG	GCG	TCC	TGT	TAC	GCC	GTA	ATA	GAG	GAC	CAG	GGG	CTT	1056
	Asp	Arg	Ile	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Ile	Glu	Asp	Gln	Gly	Leu	
				340					345					350			
85	GCG	CAT	TTG	GCC	TTC	GCG	CCC	GCC	AGG	CTC	TAT	TAT	TAC	GTG	TCA	TCA	1104

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	Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser	
	355 360 365	-
5	TTC CTG TCC CCC AAA ACT CCA GCA GTC GGT CCA ATG CGA CTT TAC AAC Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn	1152
	370 375 380	
10	AGG AGG GGG TCC ACT GGT ACT CCA GGC TCC TGT CAT CAA ATG GGA ACG Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr	1200
	385 390 395 400	
15	TGG CTT TTG GAC AGC AAC ATG CTT CAT CCT TTG GGG ATG TCA GTA AAC Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn	1248
	405 410 415	
	TCA AGC TG Ser Ser	1256
20	(2) INFORMATION FOR SEQ ID NO:6:	
	(i) SEQUENCE CHARACTERISTICS:	
25	(A) LENGTH: 1425 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
30	(ii) MOLECULE TYPE: cDNA	
	(ix) FEATURE:	
35	(A) NAME/KEY: CDS	
	(B) LOCATION: 1..1425	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:	
40	ATG CTG CTG CTG GCG AGA TGT CTG CTG CTA GTC CTC GTC TCC TCG CTG Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu	48
	1 5 10 15	
45	CTG GTA TGC TCG GGA CTG GCG TGC GGA CCG GGC AGG GGG TTC GGG AAG Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys	96
	20 25 30	
50	AGG AGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT ATC Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile	144
	35 40 45	
55	CCC AAT GTG GCC GAG AAG ACC CTA GGC GCC AGC GGA AGG TAT GAA GGG Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly	192
	50 55 60	
60	AAG ATC TCC AGA AAC TCC GAG CGA TTT AAG GAA CTC ACC CCC AAT TAC Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr	240
	65 70 75 80	
65	AAC CCC GAC ATC ATA TTT AAG GAT GAA GAA AAC ACC GGA GCG GAC AGG Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg	288
	85 90 95	
	CTG ATG ACT CAG AGG TGT AAG GAC AAG TTG AAC GCT TTG GCC ATC TCG Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser	336
	100 105 110	
	GTG ATG AAC CAG TGG CCA GGA GTG AAA CTG CGG GTG ACC GAG GGC TGG Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp	384

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	115	120	125	
5	GAC GAA GAT GGC CAC CAC TCA GAG GAG TCT CTG CAC TAC GAG GGC CGC Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg 130 135 140	432		
10	GCA GTG GAC ATC ACC ACG TCT GAC CGC GAC CGC AGC AAG TAC GGC ATG Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met 145 150 155 160	480		
15	CTG GCC CGC CTG GCG GTG GAG GCC GGC TTC GAC TGG GTG TAC TAC GAG Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu 165 170 175	528		
20	TCC AAG GCA CAT ATC CAC TGC TCG GTG AAA GCA GAG AAC TCG GTG GCG Ser Lys Ala Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala 180 185 190	576		
25	GCC AAA TCG GGA GGC TGC TTC CCG GGC TCG GCC ACG GTG CAC CTG GAG Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu 195 200 205	624		
30	CAG GGC GGC ACC AAG CTG GTG AAG GAC CTG AGC CCC GGG GAC CGC GTG Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val 210 215 220	672		
35	CTG GCG GCG GAC GAC CAG GGC CGG CTG CTC TAC AGC GAC TTC CTC ACT Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr 225 230 235 240	720		
40	TTC CTG GAC CGC GAC GAC GGC GCC AAG AAG GTC TTC TAC GTG ATC GAG Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu 245 250 255	768		
45	ACG CGG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG CTC Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu 260 265 270	816		
50	TTT GTG GCG CCG CAC AAC GAC TCG GCC ACC GGG GAG CCC GAG GCG TCC Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser 275 280 285	864		
55	TCG GGC TCG GGG CCG CCT TCC GGG GGC GCA CTG GGG CCT CGG GCG CTG Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu 290 295 300	912		
60	TTC GCC AGC CGC GTG CGC CCG GGC CAG CGC GTG TAC GTG GTG GCC GAG Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu 305 310 315 320	960		
65	CGT GAC GGG GAC CGC CGG CTC CTG CCC GCC GCT GTG CAC AGC GTG ACC Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Val His Ser Val Thr 325 330 335	1008		
70	CTA AGC GAG GAG GCC GCG GGC GCC TAC GCG CCG CTC ACG GCC CAG GGC Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly 340 345 350	1056		
75	ACC ATT CTC ATC AAC CGG GTG CTG GCC TCG TGC TAC GCG GTC ATC GAG Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu 355 360 365	1104		
80	GAG CAC AGC TGG GCG CAC CGG GCC TTC GCG CCC TTC CGC CTG GCG CAC Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His 370 375 380	1152		
85	GCG CTC CTG GCT GCA CTG GCG CCC GCG CGC ACG GAC CGC GGC GGG GAC Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp 385 390 395	1200		

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	385		390		395		400		
	AGC GGC GGC GGG GAC CGC GGG GGC GGC GGC GGC AGA GTA GCC CTA ACC							1248	
5	Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr		405		410		415		
	GCT CCA GGT GCT GCC GAC GCT CCG GGT GCG GGG GCC ACC GCG GGC ATC							1296	
	Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile		420		425		430		
10	CAC TGG TAC TCG CAG CTG CTC TAC CAA ATA GGC ACC TGG CTC CTG GAC							1344	
	His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp		435		440		445		
15	AGC GAG GCC CTG CAC CCG CTG GGC ATG GCG GTC AAG TCC AGC NNN AGC							1392	
	Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser		450		455		460		
20	CGG GGG GCC GGG GGA GGG GCG CGG GAG GGG GCC							1425	
	Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala		465		470		475		
25	(2) INFORMATION FOR SEQ ID NO:7:								
	(i) SEQUENCE CHARACTERISTICS:								
	(A) LENGTH: 1622 base pairs								
	(B) TYPE: nucleic acid								
30	(C) STRANDEDNESS: both								
	(D) TOPOLOGY: linear								
	(ii) MOLECULE TYPE: cDNA								
35	(ix) FEATURE:								
	(A) NAME/KEY: CDS								
	(B) LOCATION: 51..1283								
40	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:								
	CATCAGCCCA CCAGGAGACC TCGCCCGCCG CTCCCCGGG CTCCCCGGCC ATG TCT							56	
45							Met Ser		
							1		
	CCC GCC CGG CTC CGG CCC CGA CTG CAC TTC TGC CTG GTC CTG TTG CTG							104	
	Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu Leu		5		10		15		
50	CTG CTG GTG GTG CCC GCG GCA TGG GGC TGC GGG CCG GGT CGG GTG GTG							152	
	Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg Val Val		20		25		30		
55	GGC AGC CGC CGG CGA CCG CCA CGC AAA CTC GTG CCG CTC GCC TAC AAG							200	
	Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala Tyr Lys		35		40		45	50	
60	CAG TTC AGC CCC AAT GTG CCC GAG AAG ACC CTG GGC GCC AGC GGA CGC							248	
	Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser Gly Arg		55		60		65		
	TAT GAA GGC AAG ATC GCT CGC AGC TCC GAG CGC TTC AAG GAG CTC ACC							296	
65	Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu Leu Thr		70		75		80		
	CCC AAT TAC AAT CCA GAC ATC ATC TTC AAG GAC GAG GAG AAC ACA GGC							344	
	Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly								

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	85	90	95	
5	GCC GAC CGC CTC ATG ACC CAG CGC TGC AAG GAC CGC CTG AAC TCG CTG Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn Ser Leu 100 105 110			392
10	GCT ATC TCG GTG ATG AAC CAG TGG CCC GGT GTG AAG CTG CGG GTG ACC Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr 115 120 125 130			440
15	GAG GGC TGG GAC GAG GAC GGC CAC CAC TCA GAG GAG TCC CTG CAT TAT Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr 135 140 145			488
20	GAG GGC CGC GCG GTG GAC ATC ACC ACA TCA GAC CGC GAC CGC AAT AAG Glu Gly Arg Ala Val Asp Ile Thr Ser Asp Arg Asp Arg Asn Lys 150 155 160			536
25	TAT GGA CTG CTG GCG CGC TTG GCA GTG GAG GCC GGC TTT GAC TGG GTG Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val 165 170 175			584
30	TAT TAC GAG TCA AAG GCC CAC GTG CAT TGC TCC GTC AAG TCC GAG CAC Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser Glu His 180 185 190			632
35	TCG GCC GCA GCC AAG ACG GGC GGC TGC TTC CCT GCC GGA GCC CAG GTA Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala Gln Val 195 200 205 210			680
40	CGC CTG GAG AGT GGG GCG CGT GTG GCC TTC TCA GCC GTG AGG CCG GGA Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg Pro Gly 215 220 225			728
45	GAC CGT GTG CTG GCC ATG GGG GAG GAT GGG AGC CCC ACC TTC AGC GAT Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe Ser Asp 230 235 240			776
50	GTG CTC ATT TTC CTG GAC CGC GAG CCC CAC AGG CTG AGA GCC TTC CAG Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala Phe Gln 245 250 255			824
55	GTC ATC GAG ACT CAG GAC CCC CCA CGC CGC CTG GCA CTC ACA CCC GCT Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr Pro Ala 260 265 270			872
60	CAC CTG CTC TTT ACG GCT GAC AAT CAC ACG GAG CCG GCA GCC CGC TTC His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala Arg Phe 275 280 285 290			920
65	CGG GCC ACA TTT GCC AGC CAC GTG CAG CCT GGC CAG TAC GTG CTG GTG Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val Leu Val 295 300 305			968
70	GCT GGG GTG CCA GGC CTG CAG CCT GCC CGC GTG GCA GCT GTC TCT ACA Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val Ser Thr 310 315 320			1016
75	CAC GTG GCC CTC GGG GCC TAC GCC CCG CTC ACA AAG CAT GGG ACA CTG His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly Thr Leu 325 330 335			1064
80	GTG GTG GAG GAT GTG GTG GCA TCC TGC TTC GCG GCC GTG GCT GAC CAC Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala Asp His 340 345 350			1112
85	CAC CTG GCT CAG TTG GCC TTC TGG CCC CTG AGA CTC TTT CAC AGC TTG His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His Ser Leu			1160

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	355	360	365	370	
	GCA TGG GGC AGC TGG ACC CCG GGG GAG GGT GTG CAT TGG TAC CCC CAG				1208
5	Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr Pro Gln	375	380	385	
	CTG CTC TAC CGC CTG GGG CGT CTC CTG CTA GAA GAG GGC AGC TTC CAC				1256
10	Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser Phe His	390	395	400	
	CCA CTG GGC ATG TCC GGG GCA GGG AGC TGAAAGGACT CCACCGCTGC				1303
	Pro Leu Gly Met Ser Gly Ala Gly Ser	405	410		
15	CCTCCTGGAA CTGCTGTACT GGGTCCAGAA GCCTCTCAGC CAGGAGGGAG CTGGCCCTGG				1363
	AAGGGACCTG AGCTGGGGGA CACTGGGCTCC TGCCATCTCC TCTGCCATGA AGATACACCA				1423
20	TTGAGACTTG ACTGGGCAAC ACCAGCGTCC CCCACCCGCG TCGTGGTGTA GTCATAGAGC				1483
	TGCAAGCTGA GCTGGCGAGG GGATGGTTGT TGACCCCTCT CTCCTAGAGA CCTTGAGGCT				1543
	GGCACGGCGA CTCCCAACTC AGCCTGCTCT CACTACGAGT TTTCATACTC TGCCTCCCCC				1603
25	ATTGGGAGGG CCCATTCCC				1622
	(2) INFORMATION FOR SEQ ID NO:8:				
30	(i) SEQUENCE CHARACTERISTICS:				
	(A) LENGTH: 1251 base pairs				
	(B) TYPE: nucleic acid				
	(C) STRANDEDNESS: both				
	(D) TOPOLOGY: linear				
35	(ii) MOLECULE TYPE: cDNA				
	(ix) FEATURE:				
40	(A) NAME/KEY: CDS				
	(B) LOCATION: 1..1248				
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:				
45	ATG GAC GTA AGG CTG CAT CTG AAG CAA TTT GCT TTA CTG TGT TTT ATC				48
	Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile	1	5	10	15
50	AGC TTG CTT CTG ACG CCT TGT GGA TTA GCC TGT GGT CCT GGT AGA GGT				96
	Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly	20	25	30	
55	TAT GGA AAA CGA AGA CAC CCA AAG AAA TTA ACC CCG TTG GCT TAC AAG				144
	Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys	35	40	45	
60	CAA TTC ATC CCC AAC GTT GCT GAG AAA ACG CTT GGA GCC AGC GGC AAA				192
	Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys	50	55	60	
	TAC GAA GGC AAA ATC ACA AGG AAT TCA GAG AGA TTT AAA GAG CTG ATT				240
	Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile	65	70	75	80
65	CCG AAT TAT AAT CCC GAT ATC ATC TTT AAG GAC GAG GAA AAC ACA AAC				288
	Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn	85	90	95	

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	GCT GAC AGG CTG ATG ACC AAG CGC TGT AAG GAC AAG TTA AAT TCG TTG	336
	Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu	
	100 105 110	
5	GCC ATA TCC GTC ATG AAC CAC TGG CCC GGC GTG AAA CTG CGC GTC ACT	384
	Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr	
	115 120 125	
10	GAA GGC TGG GAT GAG GAT GGT CAC CAT TTA GAA GAA TCT TTG CAC TAT	432
	Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr	
	130 135 140	
15	GAG GGA CGG GCA GTG GAC ATC ACT ACC TCA GAC AGG GAT AAA AGC AAG	480
	Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys	
	145 150 155 160	
20	TAT GGG ATG CTA TCC AGG CTT GCA GTG GAG GCA GGA TTC GAC TGG GTC	528
	Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val	
	165 170 175	
25	TAT TAT GAA TCT AAA GCC CAC ATA CAC TGC TCT GTC AAA GCA GAA AAT	576
	Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn	
	180 185 190	
30	TCA GTG GCT GCT AAA TCA GGA GGA TGT TTT CCT GGG TCT GGG ACG GTG	624
	Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val	
	195 200 205	
35	ACA CTT GGT GAT GGG ACG AGG AAA CCC ATC AAA GAT CTT AAA GTG GGC	672
	Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly	
	210 215 220	
40	GAC CGG GTT TTG GCT GCA GAC GAG AAG GGA AAT GTC TTA ATA AGC GAC	720
	Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp	
	225 230 235 240	
45	TTT ATT ATG TTT ATA GAC CAC GAT CCG ACA ACG AGA AGG CAA TTC ATC	768
	Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile	
	245 250 255	
50	GTC ATC GAG ACG TCA GAA CCT TTC ACC AAG CTC ACC CTC ACT GCC GCG	816
	Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala	
	260 265 270	
55	CAC CTA GTT TTC GTT GGA AAC TCT TCA GCA GCT TCG GGT ATA ACA GCA	864
	His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala	
	275 280 285	
60	ACA TTT GCC AGC AAC GTG AAG CCT GGA GAT ACA GTT TTA GTG TGG GAA	912
	Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu	
	290 295 300	
65	GAC ACA TGC GAG AGC CTC AAG AGC GTT ACA GTG AAA AGG ATT TAC ACT	960
	Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr	
	305 310 315 320	
70	GAG GAG CAC GAG GGC TCT TTT GCG CCA GTC ACC GCG CAC GGA ACC ATA	1008
	Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile	
	325 330 335	
75	ATA GTG GAT CAG GTG TTG GCA TCG TGC TAC GCG GTC ATT GAG AAC CAC	1056
	Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His	
	340 345 350	
80	AAA TGG GCA CAT TGG GCT TTT GCG CCG GTC AGG TTG TGT CAC AAG CTG	1104
	Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu	
	355 360 365	

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	ATG ACG TGG CTT TTT CCG GCT CGT GAA TCA AAC GTC AAT TTT CAG GAG	1152	
	Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu		-
	370 375 380		
5	GAT GGT ATC CAC TGG TAC TCA AAT ATG CTG TTT CAC ATC GGC TCT TGG	1200	
	Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp		
	385 390 395 400		
10	CTG CTG GAC AGA GAC TCT TTC CAT CCA CTC GGG ATT TTA CAC TTA AGT	1248	
	Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser		
	405 410 415		
	TGA	1251	
15	(2) INFORMATION FOR SEQ ID NO:9:		
	(i) SEQUENCE CHARACTERISTICS:		
	(A) LENGTH: 1416 base pairs		
20	(B) TYPE: nucleic acid		
	(C) STRANDEDNESS: both		
	(D) TOPOLOGY: linear		
	(ii) MOLECULE TYPE: cDNA		
25	(ix) FEATURE:		
	(A) NAME/KEY: CDS		
	(B) LOCATION: 1..1413		
30	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:		
	ATG GAT AAC CAC AGC TCA GTG CCT TGG GCC AGT GCC GCC AGT GTC ACC	48	
35	Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr		
	1 5 10 15		
	TGT CTC TCC CTG GGA TGC CAA ATG CCA CAG TTC CAG TTC CAG TTC CAG	96	
40	Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln		
	20 25 30		
	CTC CAA ATC CGC AGC GAG CTC CAT CTC CGC AAG CCC GCA AGA AGA ACG	144	
	Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr		
	35 40 45		
45	CAA ACG ATG CGC CAC ATT GCG CAT ACG CAG CGT TGC CTC AGC AGG CTG	192	
	Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu		
	50 55 60		
50	ACC TCT CTG GTG GCC CTG CTG CTG ATC GTC TTG CCG ATG GTC TTT AGC	240	
	Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser		
	65 70 75 80		
	CCG GCT CAC AGC TGC GGT CCT GGC CGA GGA TTG GGT CGT CAT AGG GCG	288	
55	Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala		
	85 90 95		
	CGC AAC CTG TAT CCG CTG GTC CTC AAG CAG ACA ATT CCC AAT CTA TCC	336	
60	Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser		
	100 105 110		
	GAG TAC ACG AAC AGC GCC TCC GGA CCT CTG GAG GGT GTG ATC CGT CGG	384	
	Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg		
	115 120 125		
65	GAT TCG CCC AAA TTC AAG GAC CTC GTG CCC AAC TAC AAC AGG GAC ATC	432	
	Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile		
	130 135 140		

	CTT TTC CGT GAC GAG GAA GGC ACC GGA GCG GAT GGC TTG ATG AGC AAG	480	-
	Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys		
	145 150 155 160		
5	CGC TGC AAG GAG AAG CTA AAC GTG CTG GCC TAC TCG GTG ATG AAC GAA	528	
	Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu		
	165 170 175		
10	TGG CCC GGC ATC CGG CTG CTG GTC ACC GAG AGC TGG GAC GAG GAC TAC	576	
	Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr		
	180 185 190		
15	CAT CAC GGC CAG GAG TCG CTC CAC TAC GAG GGC CGA GCG GTG ACC ATT	624	
	His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile		
	195 200 205		
20	GCC ACC TCC GAT CGC GAC CAG TCC AAA TAC GGC ATG CTC GCT CGC CTG	672	
	Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu		
	210 215 220		
25	GCC GTC GAG GCT GGA TTC GAT TGG GTC TCC TAC GTC AGC AGG CGC CAC	720	
	Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His		
	225 230 235 240		
30	ATC TAC TGC TCC GTC AAG TCA GAT TCG TCG ATC AGT TCC CAC GTG CAC	768	
	Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His		
	245 250 255		
35	GGC TGC TTC ACG CCG GAG AGC ACA GCG CTG CTG GAG AGT GGA GTC CGG	816	
	Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg		
	260 265 270		
40	AAG CCG CTC GGC GAG CTC TCT ATC GGA GAT CGT GTT TTG AGC ATG ACC	864	
	Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr		
	275 280 285		
45	GCC AAC GGA CAG GCC GTC TAC AGC GAA GTG ATC CTC TTC ATG GAC CGC	912	
	Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg		
	290 295 300		
50	AAC CTC GAG CAG ATG CAA AAC TTT GTG CAG CTG CAC ACG GAC GGT GGA	960	
	Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly		
	305 310 315 320		
55	GCA GTG CTC ACG GTG ACG CCG GCT CAC CTG GTT AGC GTT TGG CAG CCC	1008	
	Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro		
	325 330 335		
60	GAG AGC CAG AAG CTC ACG TTT GTG TTT GCG CAT CGC ATC GAG GAG AAG	1056	
	Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys		
	340 345 350		
65	AAC CAG GTG CTC GTA CGG GAT GTG GAG ACG GGC GAG CTG AGG CCC CAG	1104	
	Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln		
	355 360 365		
70	CGA GTG GTC AAG TTG GGC AGT GTG CGC AGT AAG GGC GTG GTC GCG CCG	1152	
	Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro		
	370 375 380		
75	CTG ACC CGC GAG GGC ACC ATT GTG GTC AAC TCG GTG GCC GCC AGT TGC	1200	
	Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys		
	385 390 395 400		
80	TAT GCG GTG ATC AAC AGT CAG TCG CTG GCC CAC TGG GGA CTG GCT CCC	1248	
	Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro		
	405 410 415		

[illegible]

20 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 425 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

30	Met	Val	Glu	Met	Leu	Leu	Leu	Thr	Arg	Ile	Leu	Leu	Val	Gly	Phe	Ile	
	1				5					10					15		
35	Cys	Ala	Leu	Leu	Val	Ser	Ser	Gly	Leu	Thr	Cys	Gly	Pro	Gly	Arg	Gly	
				20					25					30			
	Ile	Gly	Lys	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	
			35					40					45				
40	Gln	Phe	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	
	50						55					60					
	Tyr	Glu	Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	
45	65					70					75					80	
	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	
					85					90					95		
50	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	
				100					105					110			
	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	
			115					120					125				
55	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	
	130						135					140					
	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	
60	145					150					155					160	
	Tyr	Gly	Met	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	
					165					170					175		
	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	
65				180					185					190			
	Ser	Val	Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	
			195					200					205				

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His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly
 210 215 220

5 Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp
 225 230 235 240

Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr
 245 250 255

10 Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala
 260 265 270

His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly
 275 280 285

15 Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
 290 295 300

20 Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
 305 310 315 320

Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
 325 330 335

25 Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
 340 345 350

Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
 355 360 365

Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
 370 375 380

35 Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
 385 390 395 400

Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
 405 410 415

40 Pro Leu Gly Met Val Ala Pro Ala Ser
 420 425

45 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 396 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: protein

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
 1 5 10 15

60 Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
 20 25 30

Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
 35 40 45

65 Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
 50 55 60

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Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
 65 70 75 80

5 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
 85 90 95

Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
 100 105 110

10 Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125

Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
 130 135 140

15 Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
 145 150 155 160

20 Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
 165 170 175

Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu
 180 185 190

25 Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
 195 200 205

Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
 210 215 220

Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu
 225 230 235 240

35 Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
 245 250 255

Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
 260 265 270

40 Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
 275 280 285

Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
 290 295 300

Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
 305 310 315 320

50 Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
 325 330 335

Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
 340 345 350

55 Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
 355 360 365

Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
 370 375 380

Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
 385 390 395

65

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 411 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

10 Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
 1 5 10 15
 15 Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
 20 25 30
 Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
 35 40 45
 20 Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
 50 55 60
 Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65 70 75 80
 25 Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
 85 90 95
 30 Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
 100 105 110
 Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
 115 120 125
 35 Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
 130 135 140
 His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
 145 150 155 160
 40 Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
 165 170 175
 45 Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
 180 185 190
 Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
 195 200 205
 50 Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys
 210 215 220
 Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe
 225 230 235 240
 55 Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala
 245 250 255
 60 Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
 260 265 270
 Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala
 275 280 285
 65 His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
 290 295 300
 Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val

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305 310 315 320
 Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly
 325 330 335
 5 Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
 340 345 350
 10 Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro
 355 360 365
 Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
 370 375 380
 15 Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
 385 390 395 400
 Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
 405 410
 20

(2) INFORMATION FOR SEQ ID NO:13:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 437 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

35 Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
 1 5 10 15
 40 Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
 20 25 30
 Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
 35 40 45
 45 Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
 50 55 60
 Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
 65 70 75 80
 50 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
 85 90 95
 Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
 100 105 110
 Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125
 60 Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
 130 135 140
 Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly
 145 150 155 160
 65 Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
 165 170 175

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Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val
 180 185 190
 5 Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu
 195 200 205
 Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg
 210 215 220
 10 Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu
 225 230 235 240
 Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile
 245 250 255
 15 Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu
 260 265 270
 Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser
 275 280 285
 Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val
 290 295 300
 25 Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
 305 310 315 320
 Val Thr Leu Arg Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
 325 330 335
 30 His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
 340 345 350
 Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
 355 360 365
 Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
 370 375 380
 40 Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
 385 390 395 400
 Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
 405 410 415
 45 Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
 420 425 430
 Ala Val Lys Ser Ser
 435

(2) INFORMATION FOR SEQ ID NO:14:

55 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 418 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

60 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

65 Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser
 1 5 10 15

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Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg
 20 25 30
 5 Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
 35 40 45
 Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
 50 55 60
 10 Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65 70 75 80
 Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
 85 90 95
 15 Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser
 100 105 110
 Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
 115 120 125
 Asp Glu Asp Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg
 130 135 140
 25 Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr
 145 150 155 160
 Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
 165 170 175
 30 Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
 180 185 190
 Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln
 195 200 205
 Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val
 210 215 220
 40 Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met
 225 230 235 240
 Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu
 245 250 255
 45 Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu
 260 265 270
 Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala
 275 280 285
 50 Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp
 290 295 300
 55 Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
 305 310 315 320
 Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
 325 330 335
 60 Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
 340 345 350
 65 Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
 355 360 365
 Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
 370 375 380

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Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
 385 390 395 400

5 Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
 405 410 415

Ser Ser

10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 475 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: protein

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

25 Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1 5 10 15

Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
 20 25 30

30 Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
 35 40 45

35 Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
 50 55 60

Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65 70 75 80

40 Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
 85 90 95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
 100 105 110

45 Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
 115 120 125

50 Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
 130 135 140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
 145 150 155 160

55 Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
 165 170 175

Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
 180 185 190

60 Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
 195 200 205

65 Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
 210 215 220

Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
 225 230 235 240

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Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
245 250 255 -

5 Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
260 265 270

Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser
275 280 285

10 Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu
290 295 300

15 Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu
305 310 315 320

Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr
325 330 335

20 Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly
340 345 350

Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu
355 360 365

25 Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His
370 375 380

30 Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp
385 390 395 400

Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Arg Val Ala Leu Thr
405 410 415

35 Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
420 425 430

His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
435 440 445

40 Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
450 455 460

45 Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
465 470 475

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
50 (A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Ser Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu
1 5 10 15

60 Leu Leu Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg
20 25 30

Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
35 40 45

65 Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
50 55 60

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Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65 70 75 80

5 Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
 85 90 95

Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
 100 105 110

10 Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
 115 120 125

Val Thr Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
 130 135 140

15 His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
 145 150 155 160

20 Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
 165 170 175

Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
 180 185 190

25 Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
 195 200 205

Gln Val Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg
 210 215 220

30 Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe
 225 230 235 240

35 Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala
 245 250 255

Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
 260 265 270

40 Pro Ala His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala
 275 280 285

Arg Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
 290 295 300

Leu Val Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
 305 310 315 320

50 Ser Thr His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly
 325 330 335

Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
 340 345 350

55 Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His
 355 360 365

Ser Leu Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr
 370 375 380

Pro Gln Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser
 385 390 395 400

65 Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
 405 410

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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

5

- (A) LENGTH: 416 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
 1 5 10 15

Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
 20 25 30

Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
 35 40 45

Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
 50 55 60

Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
 65 70 75 80

Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
 85 90 95

Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
 100 105 110

Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
 115 120 125

Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
 130 135 140

Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
 145 150 155 160

Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
 165 170 175

Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
 180 185 190

Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val
 195 200 205

Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly
 210 215 220

Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp
 225 230 235 240

Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile
 245 250 255

Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala
 260 265 270

His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala
 275 280 285

Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu
 290 295 300

Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65 70 75 80
 5 Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
 85 90 95
 Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
 100 105 110
 10 Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
 115 120 125
 Val Thr Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
 130 135 140
 15 His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
 145 150 155 160
 20 Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
 165 170 175
 Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
 180 185 190
 25 Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
 195 200 205
 Gln Val Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg
 210 215 220
 30 Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe
 225 230 235 240
 35 Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala
 245 250 255
 Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
 260 265 270
 40 Pro Ala His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala
 275 280 285
 Arg Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
 290 295 300
 Leu Val Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
 305 310 315 320
 50 Ser Thr His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly
 325 330 335
 Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
 340 345 350
 55 Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His
 355 360 365
 Ser Leu Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr
 370 375 380
 60 Pro Gln Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser
 385 390 395 400
 65 Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
 405 410

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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 416 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
1 5 10 15

15 Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
20 25 30

Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
35 40 45

20 Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
50 55 60

25 Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
65 70 75 80

Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
85 90 95

30 Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
100 105 110

Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
115 120 125

35 Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
130 135 140

40 Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
145 150 155 160

Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
165 170 175

45 Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
180 185 190

Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val
195 200 205

50 Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly
210 215 220

55 Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp
225 230 235 240

Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile
245 250 255

60 Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala
260 265 270

His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala
275 280 285

65 Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu
290 295 300

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Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr
 305 310 315 320
 5 Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile
 325 330 335
 Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His
 340 345 350
 10 Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu
 355 360 365
 Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu
 370 375 380
 15 Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp
 385 390 395 400
 20 Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser
 405 410 415

(2) INFORMATION FOR SEQ ID NO:18:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 471 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 30 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
 35 Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
 1 5 10 15
 Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
 20 25 30
 40 Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
 35 40 45
 Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
 50 55 60
 45 Thr Ser Leu Val Ala Leu Leu Ile Val Leu Pro Met Val Phe Ser
 65 70 75 80
 50 Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
 85 90 95
 Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
 100 105 110
 55 Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg
 115 120 125
 Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile
 130 135 140
 60 Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys
 145 150 155 160
 65 Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu
 165 170 175
 Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr
 180 185 190

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His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile
 195 200 205
 5 Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu
 210 215 220
 Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His
 225 230 235 240
 10 Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His
 245 250 255
 Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg
 260 265 270
 15 Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr
 275 280 285
 20 Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg
 290 295 300
 Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly
 305 310 315 320
 25 Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro
 325 330 335
 Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys
 340 345 350
 30 Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
 355 360 365
 35 Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
 370 375 380
 Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
 385 390 395 400
 40 Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
 405 410 415
 Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
 420 425 430
 45 Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
 435 440 445
 50 Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
 450 455 460
 Pro Gln Ser Trp Arg His Asp
 465 470
 55

(2) INFORMATION FOR SEQ ID NO:19:

- 60 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 221 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 65 (ii) MOLECULE TYPE: peptide
 (v) FRAGMENT TYPE: internal

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

5 Cys Gly Pro Gly Arg Gly Xaa Gly Xaa Arg Arg His Pro Lys Lys Leu
 1 5 10 15
 Thr Pro Leu Ala Tyr Lys Gln Phe Ile Pro Asn Val Ala Glu Lys Thr
 20 25 30
 10 Leu Gly Ala Ser Gly Arg Tyr Glu Gly Lys Ile Xaa Arg Asn Ser Glu
 35 40 45
 Arg Phe Lys Glu Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys
 50 55 60
 15 Asp Glu Glu Asn Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys
 65 70 75 80
 Asp Lys Leu Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp Pro Gly
 85 90 95
 20 Val Xaa Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly His His Xaa
 100 105 110
 Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser
 115 120 125
 25 Asp Arg Asp Xaa Ser Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala Val Glu
 130 135 140
 30 Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Lys Ala His Ile His Cys
 145 150 155 160
 Ser Val Lys Ala Glu Asn Ser Val Ala Ala Lys Ser Gly Gly Cys Phe
 165 170 175
 35 Pro Gly Ser Ala Xaa Val Xaa Leu Xaa Xaa Gly Gly Xaa Lys Xaa Val
 180 185 190
 40 Lys Asp Leu Xaa Pro Gly Asp Xaa Val Leu Ala Ala Asp Xaa Xaa Gly
 195 200 205
 Xaa Leu Xaa Xaa Ser Asp Phe Xaa Xaa Phe Xaa Asp Arg
 210 215 220

45

(2) INFORMATION FOR SEQ ID NO:20:

50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 167 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

60 Cys Gly Pro Gly Arg Gly Xaa Xaa Xaa Arg Arg Xaa Xaa Xaa Pro Lys
 1 5 10 15
 Xaa Leu Xaa Pro Leu Xaa Tyr Lys Gln Phe Xaa Pro Xaa Xaa Xaa Glu
 20 25 30
 65 Xaa Thr Leu Gly Ala Ser Gly Xaa Xaa Glu Gly Xaa Xaa Xaa Arg Xaa
 35 40 45

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Ser Glu Arg Phe Xaa Xaa Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile
 50 55 60
 5 Phe Lys Asp Glu Glu Asn Xaa Gly Ala Asp Arg Leu Met Thr Xaa Arg
 65 70 75 80
 Cys Lys Xaa Xaa Xaa Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp
 85 90 95
 10 Pro Gly Val Xaa Leu Arg Val Thr Glu Gly Xaa Asp Glu Asp Gly His
 100 105 110
 His Xaa Xaa Xaa Ser Leu His Tyr Glu Gly Arg Ala Xaa Asp Ile Thr
 115 120 125
 15 Thr Ser Asp Arg Asp Xaa Xaa Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala
 130 135 140
 20 Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Xaa Xaa His Xaa
 145 150 155 160
 His Xaa Ser Val Lys Xaa Xaa
 165

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTCCGTGGCGC CGCCGCCGCC GTCGCC

26

30 (2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

35 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

40

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

45 TTCCGATGAC CGGCCTTTCG CGGTGA

26

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(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 10 (A) DESCRIPTION: /desc = "oligonucleotide"

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

15

GTGCACGGAA AGGTGCAGGC CACACT

26

(2) INFORMATION FOR SEQ ID NO:24:

20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

30

GCGCGCTTCG AAGCGAGGCA GCCAGCGAGG GAGAGAGCGA GCGGGCGAGC CGGAGCGAGG

60

AAATCGATGC GCGC

74

(2) INFORMATION FOR SEQ ID NO:25:

35

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- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
5 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCGCGCAGAT CTGGGAAAGC GCAAGAGAGA GCGCACACGC ACACACCCGC CGCGCGCACT 60
CGGGATCCGC GCGC 74

- 15 (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 996 base pairs
(B) TYPE: nucleic acid
20 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "recombinant DNA"
25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CGAAGCGAGG CAGCCAGCGA GGGAGAGAGC GAGCGGGCGA GCCGGAGCGA GGAAATCGAA 60
GGTTCGAATC CTTCCCCCAC CACCATCACT TTCAAAAGTC CGAAAGAATC TGCTCCCTGC 120
TTGTGTGTTG GAGGTCGCTG AGTAGTGCGC GAGTAAAATT TAAGCTACAA CAAGGCAAGG 180
30 CTTGACCGAC AATTGCATGA AGAATCTGCT TAGGGTTAGG CGTTTTGCGC TGCTTCGCGA 240
TGTACGGGCC AGATATACGC GTTGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT 300
TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC TTACGGTAAA 360
TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATG ACGTCAATAA TGACGTATGT 420
TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA TGGGTGGACT ATTTACGGTA 480
35 AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT 540

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	CAATGACGGT AAATGGCCCG CCTGGCATTG TCCCCAGTAC ATGACCTTAT GGGACTTTCC	600
	TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC GGTTTTGGCA	660
	GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT	720
	TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG GACTTTCCAA AATGTCGTAA	780
5	CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG	840
	CAGAGCTCTC TGGCTAACTA GAGAACCCAC TGCTTACTGG CTTATCGAAA TTAATACGAC	900
	TCACTATAGG GAGACCCAAG CTTGGTACCG AGCTCGGATC GATCTGGGAA AGCGCAAGAG	960
	AGAGCGCACA CGCACACACC CGCCGCGCGC ACTCGG	996

We claim:

1. A method for limiting damage to neuronal cells by ischemic or epoxic conditions,
5 comprising administering to an individual a *ptc* therapeutic in an amount effective for reducing cerebral infarct volume relative to the absence of administration of the *ptc* therapeutic.
2. A method for protecting cerebral tissue of a mammal against the repercussions of ischemia which comprises administering to the mammal in need thereof a therapeutically effective
10 amount of the *ptc* therapeutic.
3. A method for the treatment of cerebral infarctions, which comprises administering to a patient in need thereof a therapeutically effective amount of the *ptc* therapeutic.
4. A method for the treatment of cerebral ischemia which comprises administering to a patient in need thereof a therapeutically effective amount of the *ptc* therapeutic.
- 15 5. A method for the treatment of stroke which comprises administering to a patient in need thereof a therapeutically effective amount of the *ptc* therapeutic.
6. A method for the treatment of transient ischemia attack which comprises administering to a patient in need thereof a therapeutically effective amount of the *ptc* therapeutic.
7. The method of any of claims 1-6, wherein the *ptc* therapeutic binds to *patched* and mimics
20 *hedgehog*-mediated *patched* signal transduction.
8. The method of claim 7, wherein the *ptc* therapeutic is a small organic molecule.
9. The method of claim 7, wherein the binding of the *ptc* therapeutic to *patched* results in upregulation of *patched* and/or *gli* expression.
10. The method of any of claims 1-6, wherein the *ptc* therapeutic is a small organic molecule
25 which interacts with neuronal cells to mimic *hedgehog*-mediated *patched* signal transduction.
11. The method of any of claims 1-6, wherein the *ptc* therapeutic mimics *hedgehog*-mediated *patched* signal transduction by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway.
- 30 12. The method of any of claims 1-6, wherein the *ptc* therapeutic alters the level of expression of a *hedgehog* protein, a *patched* protein or a protein involved in the intracellular signal transduction pathway of *patched*.
13. The method of claim 12, wherein the *ptc* therapeutic is an antisense construct which
35 inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals.

14. The method of claim 13, wherein the antisense construct is an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.

15. The method of claim 14, wherein the antisense oligonucleotide is selected from the group consisting of: 5'-GTCCTGGCGCCGCCGCCGCCGTCGCC;

5 5'-TTCCGATGACCGGCCTTTCGCGGTGA; and

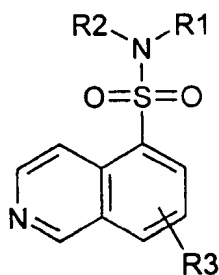
5'-GTGCACGGAAAGGTGCAGGCCACACT

16. The method of claims 12, wherein the *ptc* therapeutic is a small organic molecule which binds to *patched* and regulates *patched*-dependent gene expression.

17. The method of claim 11, wherein the *ptc* therapeutic is an inhibitor of protein kinase A.

10 18. The method of claim 17, wherein the PKA inhibitor is a 5-isoquinolinesulfonamide

19. The method of claim 18, wherein the PKA inhibitor is represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

20 R_8 , or

R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-$

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R_8 , $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$,
 $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

5 n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

20. The method of claim 17, wherein the PKA inhibitor is cyclic AMP analog.

21. The method of claim 17, wherein the PKA inhibitor is selected from the group consisting of N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat
10 Stable Inhibitor isoform α .

22. The method of claim 5, wherein the stroke is a thrombotic stroke.

23. The method of claim 5, wherein the stroke is an embolic stroke

24. The method of claim 1, wherein the hypoxic conditions result in cerebral hypoxia.

25. The method of claim 1, wherein the conditions result in progressive loss of neurons due to
15 oxygen deprivation

26. The method of any of claims 1-6, wherein patient is being treated prophylactically.

27. The method of claim 1, wherein the patient is hypotensive.

28. The method of claim 1, wherein the conditions result in progressive loss of neurons due to oxygen deprivation

20 29. The method of any of claims 1-6, wherein the *ptc* therapeutic is administered as part of a therapy including administering one or more of an anticoagulation, an antiplatelet agent, a thrombin inhibitors, and/or a thrombolytic agent.

30. The method of any of claims 1-6, wherein the *ptc* therapeutic is administered as part of a therapy including vascular surgery.

25 31. The method of claim 30, wherein the vascular surgery comprises carotid endarterectomy.

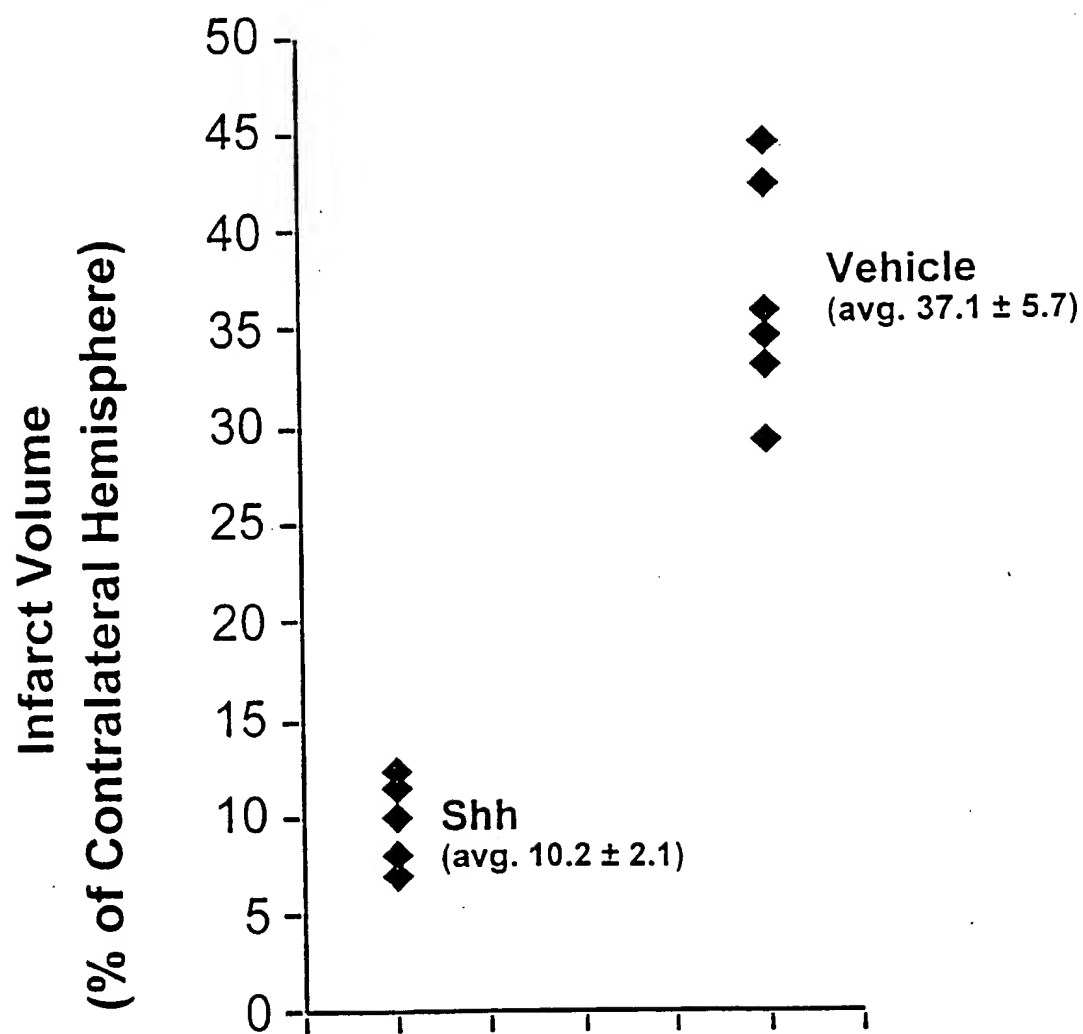
32. The method of any of claims 1-6, wherein treatment of the patient with the *ptc* therapeutic results in at least a 25% reduction in cerebral infarct volumes relative to absence of treatment with the *ptc* therapeutic.

30 33. The method of claim 32, wherein treatment of the patient with the *ptc* therapeutic results in at least a 50% reduction in cerebral infarct volumes relative to absence of treatment with the *ptc* therapeutic.

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34. The method of claim 32, wherein treatment of the patient with the *ptc* therapeutic results in at least a 70% reduction in cerebral infarct volumes relative to absence of treatment with the *ptc* therapeutic.
35. A therapeutic preparation of a small molecule antagonist of *patched*, which *patched* antagonist is provided in a pharmaceutically acceptable carrier and in an amount sufficient to provide protection against neuronal cell death under ischemic and/or hypoxic conditions.
36. The preparation of claim 35, which *patched* antagonist binds to *patched*.
37. The preparation of claim 35, wherein the *patched* antagonist is provided in an amount sufficient to produce, upon a dosage regimen of 7 days, at least a 70% decrease in infarct volume in an MCAO model relative to the absence of the *patched* antagonist.
38. The preparation of claim 37, wherein the *patched* antagonist is provided in an amount sufficient to produce, upon a dosage regimen of 3 days, at least a 70% decrease in infarct volume in an MCAO model relative to the absence of the *patched* antagonist.
39. A method for limiting damage to neuronal cells by ischemic or hypoxic conditions, comprising administering to a patient a gene activation construct which recombines with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.

1/1

Figure 1

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/47	A3	(11) International Publication Number: WO 99/00117 (43) International Publication Date: 7 January 1999 (07.01.99)
(21) International Application Number: PCT/US98/13387 (22) International Filing Date: 26 June 1998 (26.06.98) (30) Priority Data: 08/883,656 27 June 1997 (27.06.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/883,656 (CIP) Filed on 27 June 1997 (27.06.97) (71) Applicant (for all designated States except US): ONTOGENY, INC. [US/US]; 45 Moulton Street, Cambridge, MA 02138 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): MAHANTHAPPA, Nagesh, K. [US/US]; 240 Norfolk Street, Cambridge, MA 02139 (US). (74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot LLP, One Post Office Square, Boston, MA 02109 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 1 April 1999 (01.04.99)
(54) Title: NEUROPROTECTIVE METHODS AND REAGENTS		
(57) Abstract <p>One aspect of the present application relates to a method for limiting damage to neuronal cells by ischemic or epoxic conditions, e.g., such as may be manifest by a reduction in brain infarct volume, by administering to an individual a hedgehog therapeutic or ptc therapeutic in an amount effective for reducing cerabral infarct volume.</p>		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/13387

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 519 035 A (MAIESE KENNETH ET AL) 21 May 1996 see abstract see column 2, line 20-34; claims 1-5; examples 1,2 ---	1-12, 16-19, 21-28, 30-38
X	SATOH ET AL.: "Neuroprotective properties of a protein kinase inhibitor against Ischemia-induced neuronal damage in rats and gerbils" BRITISH JOURNAL OF PHARMACOLOGY, vol. 118, no. 7, 1996, pages 1592-1596, XP002092077 see abstract --- -/--	1-12, 16-19, 22-28, 30-38

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

3 February 1999

Date of mailing of the international search report

18/02/1999

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A. Jakobs

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/13387

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>MAIESE ET AL.: "Protein Kinases Modulate the Sensitivity of Hippocampal Neurons to Nitric Oxide Toxicity and Anoxia"</p> <p>JOURNAL OF NEUROSCIENCE RESEARCH, vol. 36, no. 1, 1993, pages 77-87, XP002092078</p> <p>see abstract</p> <p>see page 79, column 1, paragraph 1 - page 81, column 2, paragraph 2; figure 1</p> <p>see page 83, column 2, paragraph 2 - page 84, column 1, paragraph 3</p> <p style="text-align: center;">---</p>	<p>1-12, 16-28, 30-38</p>
X	<p>PHILLIS ET AL.: "Mechanism of glutamate and aspartate release in the ischemic rat cerebral cortex"</p> <p>BRAIN RESEARCH, vol. 730, no. 1-2, 1996, pages 150-164, XP002092079</p> <p>see abstract; figure 7; tables 2,3</p> <p style="text-align: center;">---</p>	<p>1-12, 16-28, 30-38</p>
X	<p>EP 0 187 371 A (ASAHI CHEMICAL IND ;HIDAKA HIROYOSHI (JP)) 16 July 1986</p> <p>see abstract</p> <p>see page 2, line 1-10; example 4</p> <p>see page 8, line 6 - page 14, line 4</p> <p style="text-align: center;">---</p>	<p>1-12, 16-19, 22-28, 30-38</p>
P,X	<p>WO 98 12326 A (CHUANG PAO TIEN ;HARVARD COLLEGE (US); MCMAHON ANDREW P (US)) 26 March 1998</p> <p>see page 3, line 15 - page 9, line 10; claim 13</p> <p style="text-align: center;">-----</p>	<p>1-14,40</p>

INTERNATIONAL SEARCH REPORT

I. International application No.

PCT/US 98/13387

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-34, 40
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-34, 40
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☒ Claims Nos.: -
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION SHEET PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
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3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

In view of the large number of compounds, which are defined by the general definition(s)/formulae used in claims 1-20, 22-40, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application.
(See Guidelines, chapter III, paragraph 2.3).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/13387

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5519035	A	21-05-1996	NONE	
EP 0187371	A	16-07-1986	JP 1795364 C	28-10-1993
			JP 5003851 B	18-01-1993
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			JP 61152658 A	11-07-1986
			US 4678783 A	07-07-1987
WO 9812326	A	26-03-1998	AU 4489297 A	14-04-1998